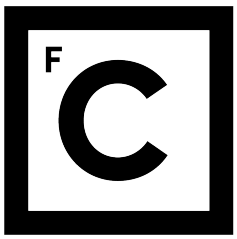
UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS



Ciências
ULisboa

Ecological, biological and molecular considerations towards the sustainable exploitation of limpets in Macaronesia (NE-Atlantic)

Doutoramento em Biologia

Especialidade em Biologia da Conservação

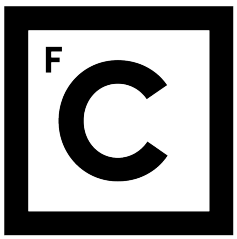
João Faria

Documento especialmente elaborado para a obtenção do grau de doutor

2018

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS



Ciências
ULisboa

Ecological, biological and molecular considerations towards the sustainable exploitation of limpets in Macaronesia (NE-Atlantic)

Doutoramento em Biologia

Especialidade em Biologia da Conservação

João Faria

Júri:

Presidente:

Doutor Henrique Manuel Roque Nogueira Cabral, Professor Catedrático, Faculdade de Ciências da Universidade de Lisboa

Vogais:

Doutora Teresa Paula Gonçalves Cruz, Professor Auxiliar, Escola de Ciências e Tecnologia da Universidade de Évora
Doutora Ana Isabel Melo Azevedo Neto, Professora Associada com Agregação, Faculdade de Ciências e Tecnologia da Universidade dos Açores
Doutora Diana Mendes Boaventura, Professora Coordenadora (equiparada), Escola Superior de Educação João de Deus
Doutor Pedro Miguel Alfaia Barcia Ré, Professor Associado com Agregação, Faculdade de Ciências da Universidade de Lisboa
Doutor José Pavão Mendes de Paula, Professor Associado com Agregação, Faculdade de Ciências da Universidade de Lisboa

Documento especialmente elaborado para a obtenção do grau de doutor

FCT – Fundação para a Ciência e Tecnologia: PTDC/BIA-BIC/ 115837/2009; PEst-C/MAR/LA0015/2013; UID/BIA/00329/2013.
Fundo Regional Ciência do Governo dos Açores: M3.1.2/ F/021/2011

2018

This dissertation should be cited as:

Faria J (2018) Ecological, biological and molecular considerations towards the sustainable exploitation of limpets in Macaronesia (NE-Atlantic). PhD Thesis, Universidade de Lisboa, Portugal

Nota Prévia:

A presente tese apresenta resultados de trabalhos já publicados ou em preparação para publicação (capítulos 2 a 7), de acordo com o previsto no nº 2 do artigo 25º do regulamento de Estudos Pós-graduados da Universidade de Lisboa, publicado no Diário de República II série nº 155 de 11 de Agosto de 2017. Tendo os trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, Dezembro de 2017

João Faria

ACKNOWLEDGMENTS

Dificilmente as palavras traduzem o verdadeiro sentimento de agradecimento a todos aqueles que, de uma forma ou de outra, contribuíram para a concretização de uma etapa de vida tão estimulante e desafiadora. A todos, o meu mais sincero agradecimento.

À Professora Doutora **Ana I. Neto** pela sua inteira disponibilidade e dedicação, muito contribuindo para o enriquecimento da minha formação académica, científica e humana. Ao Doutor **Pedro A. Ribeiro** por me acolher no seu laboratório, organizar saídas de campo e me receber na ilha do Faial, com o gim tónico do Peter a acompanhar algumas das discussões científicas. Ao Professor Doutor **Stephen J. Hawkins**, por partilhar comigo uma pequena parte da sua enorme experiência no estudo das comunidades costeiras. É uma inspiração a forma feliz e dinâmica com que continua a saltar de rocha em rocha. A eles, agradeço as discussões científicas, os valiosos comentários e sugestões na elaboração desta tese.

Aos Doutores **Pablo Presa** e **Alfonso Pita**, pela amizade e auxilio na interpretação dos dados genéticos. Ao Professor Dr. **Michael Collyer** (Western Kentucky University) pela implementação e interpretação dos dados referentes à morfometria geométrica. Ao Professor Doutor **Peter Miller** (Plymouth Marine Laboratory) e **Marc Fernandez** (GBA/cE3c – Centre for Ecology, Evolution and Environmental Changes / Azorean Biodiversity Group) por disponibilizarem dados modelados relativos às temperaturas de águas superficiais. Ao Professor Doutor **Eduardo Brito de Azevedo** (Universidade dos Açores) e **Francisco Reis** (Projecto CLIMAAT) por disponibilizarem os dados oceanográficos provenientes da boia ondógrafo localizada em Ponta Delgada, São Miguel. Ao Doutor **Luís Macedo** (Diretor Regional das Pescas dos Açores) por disponibilizar informação relativa ao número de licenças concedidas para a apanha de lapas na Região Autónoma dos Açores. Ao Doutor **Leopoldo Moro** (Governo das Canárias) pela informação prestada sobre os regulamentos de apanha de lapas nas Canárias. Ao **João Melo**, diretor do Parque Natural da Ilha do Faial, por conceder autorização para acesso e amostragem na área de Paisagem Protegida do Monte da Guia. À **Katherine Ponte**, diretora-geral do Caloura Hotel Resort, por facilitar o acesso a um dos pontos de amostragem.

A concretização desta tese não seria possível sem o auxilio e amizade de muitos aqueles que colaboraram nas saídas de campo, amostragens, trabalho de laboratório, pausas para café, discussões científicas, etc. Obrigado **Afonso Prestes**, **Arianna Cecchetti**, **Elsa Froufe**, **Emanuel Xavier**, **Eva Cacabelos**, **Fernando Tuya**, **Ignatio Moreu**, **Isadora Moniz**, **Joana Pombo**, **José Azevedo**, **Manuel Enes**, **Manuel Rivas**, **Marc Fernandez**, **Maria Vale**, **Marina Gómez**, **Marina Pastor**, **Marta Coca**, **Pedro Raposeiro**, **Rita Patarra**, **Susana Lopes**, **Zaira Nogueira**.

Não posso terminar sem um especial agradecimento ao Doutor **Gustavo Martins**. A ele, agradeço a amizade, as inúmeras conversas de café, as incursões de calhau, as ideais partilhadas, as sugestões e o entusiasmo contagiante. Foi e é uma fonte de inspiração na ciência. Não tenho dúvidas que sem ele esta tese não seria possível. Obrigado Gus.

Ao mano **André**, à família e amigos. A todos o meu profundo agradecimento.

TABLE OF CONTENTS

ACKNOWLEDGMENTS i

ABSTRACT vii

RESUMO ix

CHAPTER 1. General introduction 3

Introduction 3
Dispersal, isolation and connectivity in marine organisms 4
Recruitment in benthic marine macroinvertebrates 5
Molecular and genetic tools in connectivity studies 6
Limpets from the Macaronesia (NE Atlantic) 7
Aims, thesis outline and structure 10
References 12

CHAPTER 2. A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos 23

Introduction 24
Methods 25
Results 27
Discussion 28
Acknowledgments 29
References 30

CHAPTER 3. A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798) 35

Background 36
Samples, genetic analysis and results 36
Comments 37
Acknowledgments 37
References 37
SUPPLEMENTARY MATERIAL 38

CHAPTER 4. Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic) 45

Introduction 46
Methods 48
Results 53
Discussion 59
Acknowledgments 64
References 65
SUPPLEMENTARY MATERIAL 73

CHAPTER 5. Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): implications for conservation **83**

Introduction ... **84**
Methods ... **85**
Results ... **88**
Discussion ... **92**
Acknowledgments ... **96**
References ... **97**
SUPPLEMENTARY MATERIAL ... **104**

CHAPTER 6. Spatial and temporal patterns in recruitment in the exploited limpet *Patella candei* **117**

Introduction ... **118**
Methods ... **120**
Results ... **122**
Discussion ... **124**
Acknowledgments ... **127**
References ... **127**
SUPPLEMENTARY MATERIAL ... **134**

CHAPTER 7. Larval development of the limpet *Patella candei* at varying water temperatures: ecological implications under global warming **139**

Introduction ... **140**
Methods ... **141**
Results ... **143**
Discussion ... **145**
Acknowledgments ... **147**
References ... **149**
SUPPLEMENTARY MATERIAL ... **151**

CHAPTER 8. Overview and general discussion **157**

Discussion ... **157**
Summary of thesis findings ... **157**
Genetic diversity, population structure and connectivity of limpets in Macaronesia **158**
Recruitment and the influence of environmental variables ... **160**
Temperature influence in limpet larval development ... **162**
Implications for conservation and management ... **162**
Limitations and future perspectives ... **165**
References ... **166**

ABSTRACT

The uptake of natural living resources for human consumption has triggered serious changes in the balance of ecosystems. In the archipelagos of Macaronesia (NE Atlantic), limpets have been extensively exploited probably since islands were first colonized. This has led to profound consequences in the dynamics of rocky shore communities. The specific objectives of this thesis were to: 1) develop and characterize species-specific microsatellite markers for the limpets *Patella candei* (d'Orbigny 1840) and *Patella aspera* (Röding 1798), endemic to the Macaronesia archipelagos; 2) assess their genetic diversity, population structure and contemporary levels of connectivity throughout Macaronesia; 3) conduct a morphometric analysis of the *P. candei* complex to complement molecular data; 4) evaluate the temporal and spatial variation in recruitment of *P. candei* and study its association with real-time environmental data; 5) assess the effect of temperature on larval development of *P. candei*; and 6) provide general recommendations to foster the sustainable exploitation of limpets in Macaronesia. A total of twelve and seventeen microsatellite markers were described for *P. candei* and *P. aspera*, respectively. These showed clean polymorphisms and species-specific markers were combined in three optimized multiplex reactions. For *P. candei*, a highly significant genetic break between archipelagos following isolation by distance was detected. Contrastingly, significant genetic differentiation among islands (i.e. Azores) was absent possibly indicating ongoing gene flow via larval exchange between populations. Significant shell shape differences among archipelagos were also detected using both distance-based and geometric morphometric analyses. Adaptive processes associated with niche differentiation and strong barriers to gene flow among archipelagos may be the mechanisms underlying *P. candei* diversification in Macaronesia. As for *P. aspera*, genetic analyses showed significant population structure between populations from Azores and populations from Madeira and Canaries, and absence of current or historic gene flow between these. Results also suggest that both population clusters have experienced demographic changes over time. Heterozygote deficits were common across populations, which can be better accounted for by inbreeding than by null alleles or Wahlund effect. Such levels of inbreeding are likely a consequence of a significant reduction of reproductive units due to decades of intense exploitation. The monitoring program applied to track *P. candei* recruitment showed that early recruits occurred throughout the entire duration of the program, but its intensity varied in space and time. In general, a marked peak in recruitment occurred during winter/spring months, the period of greatest reproductive activity, when sea surface temperatures are lower and wave turbulence higher. Significant wave height was probably the most important proximate cue triggering the recruitment of *P. candei*, which eventually depends on adequate ultimate drivers for spawning and reproduction (i.e. temperature). Indeed, as a winter-breeder, *P. candei* larvae seem to perform better and attain higher fitness at colder temperatures. In fact, experimental treatments on larval rearing showed that larval development was faster at increasing temperatures but cumulative survivorship decreased; about 25% of larvae at higher temperatures survived to the end of the experiment, a 2-fold decrease from the average survivorship of ~ 50% at lower temperatures. Overall, the outcomes of this thesis fill a gap in our knowledge about processes involved in determining the connectivity patterns between limpet populations and the environmental factors influencing such patterns across the Macaronesia region.

The present study is an important first step in this direction of using multi-faceted approaches to understand complex processes operating at the marine environment, while providing a fundamental asset to define stocks and thus inform specific conservation strategies that foster the sustainable exploitation of limpets throughout Macaronesia archipelagos.

KEYWORDS: *Patella*, limpets, Macaronesia, population structure, connectivity, resilience

RESUMO

A exploração de recursos naturais biológicos para o consumo humano tem provocado alterações graves no equilíbrio dos ecossistemas. Nos arquipélagos da Macaronésia (NE Atlântico), as lapas têm sido extensivamente exploradas, provavelmente desde que as ilhas foram colonizadas. Isso levou a profundas consequências na dinâmica das comunidades litorais. Os objetivos específicos desta tese foram: 1) desenvolver e caracterizar marcadores de microssatélites específicos para as espécies de lapas *Patella candei* (d'Orbigny 1840) e *Patella aspera* (Röding 1798); 2) avaliar a sua diversidade genética, estrutura populacional e conectividade na Macaronésia; 3) realizar uma análise morfométrica do complexo de espécies *P. candei* para complementar os dados moleculares; 4) avaliar a variação temporal e espacial no recrutamento de *P. candei* e estudar sua associação com dados ambientais; 5) avaliar o efeito da temperatura no desenvolvimento larvar de *P. candei*; e 6) fornecer recomendações gerais para promover a exploração sustentável de lapas na Macaronésia. Um total de doze e dezassete marcadores de microssatélites foram caracterizados para *P. candei* e *P. aspera*, respetivamente, e amplificados em reações multiplex devidamente otimizadas. Para *P. candei*, foi detetada uma diferenciação genética significativa entre os arquipélagos. Ao invés, não se verificou diferenciação entre ilhas (isto é, Açores), o que constitui provavelmente um sinal da existência de fluxo migratório de indivíduos (larvas) entre populações. Foram também detetadas diferenças significativas na forma da concha entre os arquipélagos. Processos adaptativos associados à variabilidade de nichos e fortes barreiras ao fluxo genético entre os arquipélagos podem constituir os mecanismos subjacentes à diversificação de *P. candei* na Macaronésia. Quanto a *P. aspera*, as análises genéticas mostraram uma diferenciação populacional entre populações dos Açores e as populações da Madeira e Canárias. Os resultados também sugerem que ambos os grupos populacionais sofreram mudanças demográficas ao longo do tempo. Os défices heterozigóticos revelaram-se comuns em todas as populações, provavelmente uma consequência de processos endogâmicos associados a uma redução significativa dos indivíduos reprodutores, devido a décadas de exploração intensa. O programa de monitorização do recrutamento de *P. candei* permitiu verificar que surgem novos recrutas durante todo o ano, embora a sua abundância varie no espaço e no tempo. Em geral, um pico acentuado ocorre durante os meses de inverno/ primavera, coincidente com o período de maior atividade reprodutora, no qual as temperaturas da superfície da água do mar são baixas e é maior a agitação marítima. A par da temperatura (sobretudo pelo seu papel na atividade reprodutora), a altura significativa das ondas é provavelmente o fator mais importante a desencadear o recrutamento de *P. candei*. Por outro lado, os dados de desenvolvimento larvar revelaram que o desenvolvimento é mais acelerado a temperaturas superiores, mas a sobrevivência acumulada diminuiu; cerca de 25% das larvas expostas a temperaturas mais elevadas sobreviveram até ao final da experiência, sendo que cerca de metade das larvas sobreviveu a temperaturas inferiores. Em geral, os resultados desta tese preenchem uma lacuna no conhecimento sobre os processos envolvidos na determinação dos padrões de conectividade entre populações de lapas e os fatores ambientais que influenciam esses padrões na região da Macaronésia. O presente estudo constitui um importante primeiro passo no uso de abordagens multidisciplinares para compreender processos complexos que operam no meio marinho, proporcionando uma ferramenta

fundamental para definir stocks e assim propor estratégias de conservação específicas que promovam uma exploração sustentável de lapas em toda a Macaronésia.

PALAVRAS-CHAVE: *Patella*, lapas, Macaronesia, estrutura populacional, conectividade, resiliência

CHAPTER 1

General introduction

Introduction

There is growing consensus that anthropogenic activities are impacting the structure and functioning of marine ecosystems. For instance, over-fishing is known to have profound community-level effects particularly when keystone species are targeted (Durán and Castilla 1989; McClanahan *et al.* 1996; Orensanz *et al.* 1998; Jackson *et al.* 2001; Ainley and Blight 2009; Smith *et al.* 2011). This is especially relevant in oceanic archipelagos such as Macaronesia, which present unique habitats with fragile communities that are highly susceptible to degradation and ecosystem disruption (Hawkins *et al.* 2000). Potential factors contributing to such fragility include the generally low and stochastic nature of benthic recruitment in a dispersive island environment (Siegel *et al.* 2008) and the often frequent and pervasive nature of human resource exploitation.

For the last decades, perhaps centuries, rocky shore limpets have been heavily exploited throughout Macaronesia, and contemporary stocks are facing a continuous risk of overexploitation (Santos *et al.* 1990, 1995; Morton *et al.* 1998; Hawkins *et al.* 2000; Martins *et al.* 2008). One of the greatest challenges in fisheries research is the adequate delimitation and identification of such stocks (e.g. Hawkins *et al.* 2016). Failure by fishery managers to account for stock complexity and composition may lead to the depletion of particular components, with unknown ecological consequences (Stephenson 1999). Stock delimitation should be able to integrate data from multiple sources, and use genetic as well as physical, biological and ecological information to identify the demographic processes involved in the population dynamics of a given exploited species. Genetic approaches can give insights into the broad definition and extent of genomic admixture among management units, which are important in conservation efforts especially in overexploited species at risk of extinction. These molecular tools can expand our ability to understand population demography, dynamics and structure, while uncovering putative adaptive genetic differentiation among populations and/or species. Population genomics can thus provide vital information for fisheries management and species conservation, as it can potentially revolutionize the delineation of conservation units, as well as our understanding of the adaptive response of populations to rapid environmental changes and the evolutionary consequences of selective harvesting (Nielsen *et al.* 2009; Funk *et al.* 2012). On its own, however, genetic approaches may be insufficient to quantify true levels of demographic connectivity and the ecological effects of movement among populations of a given species (Turner *et al.* 2002; Chapuis *et al.* 2011; Hawkins *et al.* 2016). Information on life history traits, such as reproduction, development, growth, transport and fate of planktonic larvae, settlement and recruitment, provides additional insights into larval dispersal and ecological population connectivity, and hence, a more accurate understanding of population dynamics.

This thesis addresses the population genetics and dynamics of two exploited species of limpets in Macaronesia in general and the Azores in particular. The overall aim is to delimit stock units to enable management and conservation of these economically and culturally important species. In addition to population genomic approaches using next generation sequencing, basic biological and ecological information essential to understand connectivity and population dynamics has been collected. Below, I briefly review the literature on marine dispersal and connectivity, recruitment in benthic invertebrates, the use of molecular genetic tools to understand connectivity, and Macaronesian limpets, before outlining the overall aims and rationale of the thesis.

Dispersal, isolation and connectivity in marine organisms

Many sessile marine organisms have a life-history cycle which includes a pelagic larval stage with high potential for dispersal (Cowen *et al.* 2000; Morgan 2001). This has driven researchers to accept that marine populations can be regarded as open, meaning that their offspring are freely spreading and mixing over large geographical areas (Cowen *et al.* 2000). However, this traditional view has often been challenged by a number of studies who have reported far more restricted dispersal than previously predicted, suggesting that a large proportion of larvae may be retained and do not take advantage of their full putative potential for dispersal (Jones *et al.* 1999; Cowen *et al.* 2000; Mora and Sale 2002; Swearer *et al.* 2002; Warner and Cowen 2002; Levin 2006; Cowen *et al.* 2006; Wood and Gardner 2007; Gaines *et al.* 2007; Jenkins *et al.* 2007). Evidence of limited dispersal have been provided by i) the use of novel molecular, genetic and geochemical techniques (Almany *et al.* 2007; Becker *et al.* 2007; Teske *et al.* 2011); ii) the recognition of the significance of many biological traits, such as larval behaviour and open-water mortality (Burton and Feldman 1982; Fisher 2005; Sanford *et al.* 2006); and iii) a better understanding of physical processes that restrict larval movement, such as oceanographic dominant currents, eddies and upwelling systems (Thornhill *et al.* 2008; Galarza *et al.* 2009). In fact, the connectivity and structuring of marine populations result from several biological (life history traits, larval behaviour), ecological (food availability, species interactions), and physical (past and present oceanographic and climatic features) processes (see Grantham *et al.* 2003). The interplay of these factors, acting across a range of spatial and temporal scales, has significant implications in determining the amount of gene flow and connectivity between usually patchily distributed marine populations (Palumbi 1994; Cowen and Sponaugle 2009 and references therein). Occasionally, populations may become isolated if factors negatively affecting dispersal are substantial in time and space, with migration or dispersal becoming fully restricted. Under such scenario, isolated populations slowly differentiate and adapt to the point of reproductive isolation and new different species may be generated (Allendorf and Luikart 2007).

On top of all factors affecting dispersion, geographic distance between areas of suitable habitat can by itself promote population isolation such that no or only limited contact is possible between them (Avise 2001). This is particularly important on oceanic islands, which provide a good example of isolation by distance leading to speciation. Oceanic islands, by definition, have never been connected to any landmass. All the species present on those islands colonized them via exceptional events of long

distance dispersal, and then diverged from their mainland source populations, sometimes into new endemic species. Additionally, evidence suggests that self-recruitment increases with isolation and that isolated islands will on average receive lowers levels of recruitment, owing to dispersive larval loss, than equivalent mainland sites (Cowen *et al.* 2000); this is notable even on islands close to mainland (e.g. Crisp and Southward 1958; Hawkins and Hiscock 1983).

Given the intrinsic difficulties of tracking marine particles in their natural environment, processes affecting larval dispersal and, hence, population connectivity, are still poorly understood (Swearer *et al.* 2002; Weersing and Toonen 2009). In fact, complex interactions between coastal geomorphology, ocean currents and species life-history traits result in variable and complicated temporal and spatial patterns of larval dispersion and settlement (e.g. Lagos *et al.* 2008). For example, there is now mounting evidence that connectivity and gene exchange between populations can be fairly low, even in species that can freely disperse their offspring. For instance, gene flow was shown to be restricted for species with (*Semibalanus balanoides*), but also without (*Nucella lapillus*) planktonic larval stages (Bell 2008). A meta-analysis study by Weersing and Toonen (2009) revealed that the capacity to disperse (provided by the pelagic larval duration) does not predict the magnitude of gene flow and geographic scale of population structure in marine systems. This suggests that, in many cases, larval exchange is restricted despite the inherent potential to disperse (Hedgecock 1986). Low levels of larval exchange may thus limit the success of any protected area and may prevent multiple conservation objectives from being achieved (Cowen *et al.* 2006). Understanding the scale at which populations are connected is therefore a fundamental aspect to consider in biodiversity conservation, stock delimitation, fishery management, and the spatial design of marine protected areas. Indeed, many theoretical studies suggest that population connectivity plays a fundamental role in local and metapopulation dynamics, community dynamics and structure, genetic diversity, and the resiliency of populations to human exploitation (for overviews see Hansson 1991; Hastings and Harrison 1994; Botsford *et al.* 2009).

Recruitment in benthic marine macroinvertebrates

Recruitment variability remains one of the most striking features of the dynamics of many marine fish (e.g. Hjort 1914; Lasker 1981; Cushing 1990) and invertebrate populations (e.g. Bowman and Lewis 1977; Hawkins and Hartnoll 1982; Gaines *et al.* 1985; Caffey 1985; Roughgarden *et al.* 1988; Underwood and Fairweather 1989) and it is often a direct consequence of the life history strategy of many marine species (Fogarty *et al.* 1991). Recruitment, defined as the addition of new individuals to a population, is indeed a major aspect of population dynamics and it is intrinsically related to concepts such as dispersal and population connectivity (Cowen *et al.* 2006). At the marine realm it encompasses a range of stages and processes that are affected by a suite of biotic and abiotic factors, often operating at very different temporal and spatial scales (Caley *et al.* 1996). Although environmental variability is the dominant source of short-term fluctuations in recruitment, the longer-term dynamics are regulated by the interaction between environmental stochasticity and modes of population regulation (Fogarty *et al.* 1991).

Most marine invertebrates are relatively sessile or sedentary as adults but have an obligate pelagic larval stage that is dispersed by ocean currents while it develops competency to settle (Marshall and Morgan 2011). From the moment adult individuals reproduce, until juveniles are added to the population, early life stages (and even gametes if fertilization is external) are subject to the interactive role of many physical, chemical and biological factors that can potentially affect the growth and survival of these free-living units. These include i) variation in the reproductive output of adult populations which determines the abundance and quality of propagules in the water column (Uriz *et al.* 1998; Treml *et al.* 2012); ii) trophic interactions such as competition, predation, the presence of suitable prey, diseases, parasitism which can all act to determine the fate of early life-stages in a highly dynamic environment (Keough and Downes 1982; Murphy *et al.* 2014; Gagliano *et al.* 2007; Spounagle *et al.* 2011); iii) variable offshore and nearshore oceanographic processes with distinct physical attributes that can either limit or enhance the movement, development and onshore transport of planktonic larvae (Hawkins and Hartnoll 1982; Pineda *et al.* 2007; Morgan *et al.* 2009; Baltazar-Soares *et al.* 2014); iv) planktonic larval behaviour and choices made by larvae at the time of settlement (Crisp 1955; Jenkins 2005; Murphy *et al.* 2014); and v) availability of suitable sites to settle and recruit (Gaines and Bertness 1992). Indeed, recruits may vary in their settlement/post-settlement fitness depending on their source, genetic composition and/or experiences during the larval stage (e.g. Schmidt and Rand 2001).

Recruitment exhibits therefore a highly variable pattern, being determined by a set of interacting processes working upon the development, transport, settlement and post-settlement of early-life stages of marine invertebrates. Understanding the factors that affect the dispersal of propagules, their mortality rates, the recruitment output, the degree of connectivity between local populations, the phylogeographic structure and the impacts of local extinction, either natural or anthropogenic-derived, on the resilience of marine populations holds particular importance in biological conservation. This challenging task is essential for an ecosystemic approach to the management of marine environment, not only for addressing short-term management issues but also to predict the potential effects of global change on species and systems.

Molecular and genetic tools in connectivity studies

Genetic studies can make an important contribution for the understanding of population dynamics by allowing us to test hypothesis about larval dispersal, population connectivity, genetic diversity, and population equilibrium. Comprehensive reviews of different genetic marker types have been previously reported (Avise 1994; Sunnucks 2000; Schlötterer 2004), as were the advantages of microsatellites over other DNA markers (Schlötterer 2000; Selkoe and Toonen 2006). Microsatellites or simple sequence repeats are tandemly repeated motifs of 1 - 6 bases found in all prokaryotic and eukaryotic genomes analysed to date. High degree of length polymorphism and the relative ease of scoring the alleles represent two major features that make microsatellites of exceptional interest in genetic studies (Zane *et al.* 2002). Their high variability makes them very powerful genetic markers and they have proven to be an extremely useful tool for a range of applications including ecological and evolutionary

studies, genomic mapping, pedigree analysis and investigations of the genetic structure of populations (for review see Jarne and Lagoda 1996). The use of genetic information has also become a popular tool in conservation studies (Wolf *et al.* 2000; Salgueiro *et al.* 2008), with only a few focusing on commercially important or exploited species (e.g. Knutsen *et al.* 2003). Overall, fast-evolving markers such as microsatellites can increase our understanding of the population genetic structure in marine organisms and of the role of their reproductive strategies. It can also highlight some concepts such as population connectivity, gene flow, recruitment, larval dispersal, or contemporary demographic history of populations and thus providing important information for conservation and management strategies (Weersing and Toonen 2009).

To date, few studies have focused on the genetic structure of patellid limpets from the Macaronesia archipelagos (e.g. Côrte-Real *et al.* 1996; Weber and Hawkins 2005, 2006; Sá-Pinto *et al.* 2005, 2008). These focused mostly on phylogeny and/or inter-specific relationships among limpets, and in most cases, the use of allozymes, mitochondrial DNA, and/or nuclear genes, did not show the necessary resolution to address questions about structuring processes, especially at intra-species levels. Even so, despite relatively conflicting results about the phylogenetic relationships within the genus *Patella* in the Macaronesia, all studies agree and point out for clear limitations in gene flow between the Macaronesian islands and the adjacent continental platforms. As previously mentioned, although microsatellites are increasingly recognized as an efficient and high-resolution genetic tool, and have been used in patellids limpets before (see Ribeiro *et al.* 2010), no study has yet used them to examine the genetic structure of patellid limpets throughout Macaronesia.

Limpets from the Macaronesia (NE Atlantic)

Located in the Northeast Atlantic, the Macaronesia region consists of five archipelagos: Azores, Madeira, Selvagens, Canaries and Cape Verde (Fig. 1). The region is defined as a biogeographic entity based on the similarities found among archipelagos in terms of fauna and flora. All islands are of volcanic origin and have distinct but fairly recent geological age (see Ávila *et al.* 2016). Patellid limpets inhabiting these archipelagos are considered a valuable resource and have been intensively exploited presumably since islands were first colonized (Santos *et al.* 1995; Côrte-Real *et al.* 1996; Hawkins *et al.* 2000; Navarro *et al.* 2005).

Given that active grazing by limpets is a key process in shaping the structure and functioning of rocky shore communities (Underwood 1980; Branch 1981; Hawkins and Hartnoll 1983; Dungan 1986; Menge 2000; Boaventura *et al.* 2002; Jenkins *et al.* 2005; Jonsson *et al.* 2006; Davies *et al.* 2007; for meta analysis of marine herbivory impacts see Poore *et al.* 2012), the over-exploitation of limpet populations can have a large impact on the ecosystem. For instance, Martins *et al.* (2008) have shown that exploitation of limpets in Azores has pushed the intertidal community to an alternate state where turf-forming algae have displaced barnacles as the dominant space occupier resulting in shifting the structure and functioning of this ecosystem. In fact, under reduced numbers of limpets, algal sporelings and germlings can opportunistically grow to a size that allows them to escape grazing and

thus form mature algal patches that are able to persist through time altering the community dynamics and energy flow (Hawkins *et al.* 1992; Coleman *et al.* 2006).

Figure 1. Location of Macaronesia archipelagos: Azores, Madeira, Canaries and Cape Verde.

In the Azores, limpets represented an important economic resource up to the 1980's, when they were the 6th most profitable regional fishery (Martins *et al.* 1987a). In 1988 the limpet fishery in São Miguel, Azores, collapsed, and after a one-year ban throughout the archipelago, the stocks were allowed to recover, avoiding catastrophic over-exploitation effects (Hawkins *et al.* 2000). In 1993, legislation was passed to protect limpet populations. Limpet no-take areas were created and seasonal harvesting restrictions were applied in addition to minimum legal catch sizes (Decreto Regulamentar Regional nº 14/93/A). A relatively recent survey of limpet populations in Azores has, however, detected clear signs of over-exploitation with populations virtually extinct in some islands (Martins *et al.* 2008).

Patellid limpets are broadcast spawners and go through a planktonic larval stage in their life cycle. Adult animals are benthic and the larva is the only phase during their life-cycle which has the ability to disperse over large spatial scales. In dispersive isolated oceanic islands such as Macaronesia islands, it is not clear whether the different island populations form a single metapopulation (a common larval pool to each archipelago) or, in contrast, populations on each island are isolated from other populations. This, however, has important implications for the management and conservation of exploited stocks.

Two well-known limpet species occur simultaneously across the archipelagos of Azores, Madeira (including Selvagens) and Canaries; these are *Patella candei* (d'Orbigny 1840) and *Patella aspera* (Röding 1798) (Fig. 2). The former is usually found at mid and high shore and also subtidally, especially on boulders (Hawkins *et al.* 2000); the later occurs in the low intertidal and to 10 m depths

(Hawkins *et al.* 1990a); its distribution is not usually continuous, but interrupted by an algal turf in the lower eulittoral to shallow sublittoral (Hawkins *et al.* 1990b). In addition, *P. piperata* occurs in the Canaries and Madeira, but not the Azores (Christiaens 1973).

Figure 2. Representative images (A and B) and illustrations (C) of *Patella aspera* (left) and *Patella candei* (right). Ilustrations by Les Gallagher - Fishpics® & IMAR-DOP, University of the Azores.

In the past decades, the taxonomy of the *P. candei* species complex across Macaronesia has received a fair amount of attention (see Christiaens 1973; Côrte-Real *et al.* 1996; Weber and Hawkins 2002; Sá-Pinto *et al.* 2005). For instance, a comprehensive taxonomic review of the genus *Patella* has assigned a particular sub-species to a specific archipelago, i.e. *P. candei gomesii* found throughout the Azores, *P. candei ordinaria* in Madeira, *P. candei crenata* in the Canaries, and *P. candei candei* in the Selvagens Islands and Fuerteventura island (for revision see Christiaens 1973). Subsequent work using allozymes showed that *P. candei* from the Azores differed from *P. candei* in Madeira and Canaries (Côrte-Real *et al.* 1996). Allozyme electrophoresis was also used to study populations of *P. candei* from the Macaronesian archipelagos (Weber and Hawkins 2002) and two distinct genetic groups were found, one comprising *P. candei gomesii* and *P. candei candei* and the other combining *P. candei crenata* and *P. candei ordinaria*. A suggestion was made that these two groups should be considered as two subspecies of *P. candei*, based on intermediate phenotypes found in the Canaries and Selvagens. More recent work done by Sá-Pinto *et al.* (2005) did not fully support previous findings. In their study, and using modern mtDNA techniques, two well-differentiated groups were always identified in the Macaronesian islands: one including *P. candei* from the Azores, Desertas and Madeira, and another one grouping *P. candei* from the Canaries and Selvagens together with *Patella lugubris* from Cape Verde. Given such confliting results, there is still some uncertainty as to whether *P. candei* from the different archipelagos should be given a specific status or not.

Without much taxonomic debate, and formerly known as *P. ulyssiponensis* (which is now the name given for continental specimens), the designation of *P. aspera* arose from the detection of genetic differences between individuals from Macaronesia islands and those from continental Europe (Weber and Hawkins 2005). Whilst *P. candei* is a typical gonochoristic species, *P. aspera* is a protandric sequential hermaphrodite species with external fertilization, where individuals start off as males but may undergo a sex change with age (Martins *et al.* 1987b). Hence exploitation tends to focus on the larger females in the population as larger limpets (predominantly females) are selectively removed (Martins *et al.* 2017). Despite conservation legislation in the region, *P. aspera* limpets are under severe pressure and few individuals survive long enough to become females, a phenomenon that severely restricts the effective population size (Allendorf *et al.* 2008), although some compensation is apparent due to density dependent processes (Martins *et al.* 2017; see also Borges *et al.* 2016 and Fenberg and Roy 2012 for work on the protandrous limpets *Patella vulgata* and *Lottia gigantea*, respectively).

Aims, thesis outline and structure

This thesis is focused on limpets because they are an important resource, but also ecologically important species that require prioritizing conservation strategies. There is a clear need for work on limpets in the Macaronesia, prompted by the fact that these species are highly exploited commercially and their stocks in most islands have been declining steadily with profound negative effects on coastal communities. One of the main goals of this thesis is to evaluate the genetic diversity and structure of limpet populations, and hence, connectivity throughout the region, expecting that results can help

identify stocks and conservation units with potential benefits for management plans targeting the preservation of limpets across the region.

Overall, the following key questions related to limpet population dynamics in Macaronesia are addressed: are populations inhabiting different islands connected; if so, to what extent; and what is the spatial scale of gene flow, and when does it become restricted? Are there areas of higher genetic diversity that require prioritizing and special protection? What is the impact of human exploitation on the genetic diversity and structure of limpets? What factors contribute to the recruitment and realised dispersal of limpets throughout the region? Are limpet populations sufficiently resilient to withstand fishing pressure, given their isolation on oceanic islands? What recommendations can be made to promote better sustainable practices in limpet harvesting?

The thesis will focus only on the limpet species occurring in the Azores archipelago, where the core of experimental work was performed: *Patella candei* and *P. aspera*.

Starting with the development and characterization of high-resolution, species-specific microsatellite markers [Chapter 2 (Faria *et al.* 2016) and Chapter 3 (Faria *et al.* 2015)], this thesis reports patterns of genetic diversity and structure for both *P. candei* and *P. aspera* throughout the Macaronesia region [Chapter 4 (Faria *et al.* 2017a) and Chapter 5 (Faria *et al.* 2017b), respectively]. Given that the addition of new individuals to populations is a direct consequence of the realised dispersal capacity of a species, the spatial and temporal recruitment patterns of limpets and their relationship with environmental variables will then be described and discussed (Chapter 6). Given that *P. aspera* is known to recruit mostly below low tides, and ocean conditions in the Azores are rather severe during wintertime preventing adequate sampling, monitoring of recruitment was only possible for *P. candei*, which is mostly distributed at mid-shore levels, providing a relatively easier way to access sampling sites. This research was complemented with a temperature-controlled larval rearing experiment, since it is known that temperature is the main factor affecting the development and duration of larval stages in open water, and therefore influences the capacity of larvae to disperse (Chapter 7). A final general discussion integrating the results of all chapters is provided in Chapter 8. This final chapter includes also implications to the studied species conservation and management, and suggestions for future research. The structure and original content of the published papers (Chapters 2 – 5) were maintained and only scientific style and format of references, figures and tables were changed to provide consistency and standardization to the dissertation as a whole.

Overall, the genetic data, alongside with biological, ecological and oceanographic information, produced, gathered and/or discussed in this thesis, are expected to make an important contribution for the understanding of population dynamics of limpets in Macaronesia by allowing testing hypothesis about larval dispersal patterns, recruitment and life history traits, population connectivity, genetic diversity, and resilience of limpet populations to human exploitation. Due to its multidisciplinary approach, this PhD thesis will provide information of theoretical as well as practical importance that may be used to inform conservation strategies and promote the sustainable exploitation of limpets in Macaronesia.

References

Ainley DG, Blight LK (2009) Ecological repercussions of historical fish extraction from the Southern Ocean. *Fish and Fisheries* 10: 13-38.

Allendorf FW, England PR, Luikart G, Ritchie PA Ryman N (2008) Genetic effects of harvest on wild animal populations. *Trends in Ecology and Evolution* 23: 327-337.

Allendorf FW, Luikart G (2007) *Conservation and the genetics of populations*. Blackwell Publishing, London.

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. *Science* 316: 742-744.

Ávila S, Melo C Berning B, Cordeiro R Landau B, da Silva CM (2016) *Persististrombus coronatus* (Mollusca: Strombidae) in the lower Pliocene of Santa Maria Island (Azores, NE Atlantic): Paleoecology, paleoclimatology and paleobiogeographic implications. *Palaeogeography, Palaeoclimatology, Palaeoecology* 441: 912-923.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.

Avise JC (2001) *Phylogeography, the history and formation of species*. Harvard University Press, Cambridge, MA.

Baltazar-Soares M, Biastoch A, Harrod C, Hanel R, Marohn L, Prigge E, Evans D, Bodles K, Behrens E, Boning CW, Eizaguirre C (2014) Recruitment collapse and population structure of the european eel shaped by local ocean current dynamics. *Current Biology* 24: 104-108.

Becker BJ, Levin LA, Fodrie FJ, McMillan PA (2007) Complex larval connectivity patterns among marine invertebrate populations. *Proceedings of the National Academy of Sciences of the USA* 104: 3267-3272.

Bell JJ (2008) Connectivity between island Marine Protected Areas and the mainland. *Biological Monographs* 141: 2807-2820.

Boaventura D, Alexander M, Santina PD, Smith ND, Ré P, Fonseca LC, Hawkins SJ (2002) The effects of grazing on the distribution and composition of low-shore algal communities on the central coast of Portugal and on the southern coast of Britain. *Journal of Experimental Marine Ecology and Biology 2*67: 185-206.

Borges CDG, Hawkins SJ, Crowe TP, Doncaster CP (2016) The influence of simulated exploitation on *Patella vulgata* populations: protandric sex change is size-dependent. *Ecology and Evolution* 6(2): 514-531.

Botsford LW, White JW, Coffroth MA, Paris CB, Planes S, Shearer TL, Thorrold SR Jones GP (2009) Connectivity and resilience of coral reef metapopulations in marine protected areas: matching empirical efforts to predictive needs. *Coral Reefs* 28: 327-337.

Bowman RS, Lewis JR (1977) Annual fluctuations in the recruitment of *Patella vulgata* L. *Journal of the Marine Biological Association of the UK* 57: 793-815.

Branch GM (1981) The biology of limpets: physical factors, energy flow, and ecological interactions. *Oceanography and Marine Biology: an annual Review* 19: 235-380.

Burton RS, Feldman MW (1982) Population genetics of coastal and estuarine invertebrates: does larval behavior influence population structure?. In *Estuarine Comparisons.* Kennedy VS (Eds). Academic Press, New York. pp. 537-551.

Caffey HM (1985) Spatial and temporal variation in settlement and recruitment of intertidal barnacles. *Ecological Monographs* 55: 313- 332.

Caley MJ, Carr MH, Hixon MA, Hughes TP, Jones GP, Menge BA (1996) Recruitment and the local dynamics of open marine populations. *Annual Review of Ecology and Systematics* 27: 477-500.

Chapuis MP, Popple JAM, Berthier K, Simpson SJ, Deveson E, Spurgin P, Steinbauer MJ, Sword GA (2011) Challenges to assessing connectivity between massive populations of the Australian plague locust. *Proceedings of the Royal Society B, Biological Sciences* 278: 3152-3160.

Christiaens J (1973) Révision du genre *Patella* (Mollusca, Gastropoda). *Bulletin du Muséum National D'histoire Naturelle* 182: 1305-1392.

Coleman RA, Underwood AJ, Benedetti-Cecchi, Aberg P, Arenas F, Arrontes J, Castro J, Hartnoll RG, Jenkins SR, Paula J, Santina PD, Hawkins SJ (2006) A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia* 147: 556-564.

Côrte-Real HBSM, Hawkins SJ, Thorpe JP (1996) Population differentiation and taxonomic status of the exploited limpet *Patella candei* in the Macaronesian Islands (Azores, Madeira, Canaries). *Marine Biology* 125: 141-152.

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of marine populations: open or closed?. *Science* 287: 857-859.

Cowen RK, Paris CB, Srinivasan A (2006) Scaling connectivity in marine populations. *Science* 311: 522-527.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review of Marine Science* 1: 443-466.

Crisp DJ (1955) The behaviour of barnacle cyprids in relation to water movement over a surface. *Journal of Experimental Biology* 32: 569-590.

Crisp DJ, Southward AJ (1958) The distribution of intertidal organisms along the coasts of the English Channel. *Journal of the Marine Biological Association of the UK* 37: 157-208.

Cushing DH (1990) Plankton production and year-class strength in fish populations: an update of the match/mismatch hypothesis. *Advances in Marine Biology* 26: 249-293.

Davies AJ, Johnson MP, Maggs CA (2007) Limpet grazing and loss of *Ascophyllum nodosum* canopies on decadal time scales. *Marine Ecology Progress Series* 339: 131-141.

Decreto Regulamentar Regional nº 14/93/A. *Regulamento da apanha de lapas*. Governo Regional dos Açores.

Dungan ML (1986) Three-way interactions: barnacles, limpets, and algae in a Sonoran Desert rocky intertidal zone. *American Naturalist* 127: 292-316.

Durán LR, Castilla JC (1989) Variation and persistence of the middle rocky intertidal community of central Chile, with and without human harvesting. *Marine Biology* 103: 555-562.

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46(1): 96-97.

Faria J, Pita A, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Neto AI, Presa P (2016) A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 775-781.

Faria J, Martins GM, Pita A, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017a) Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic). *Ecology and Evolution* 7(16): 6125-6140.

Faria J, Pita A, Martins GM, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017b) Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): implications for conservation. *Fisheries Research* 198: 180-188.

Fenberg PB, Roy K (2012) Anthropogenic harvesting pressure and changes in life history: insights from a rocky intertidal limpet. *American Naturalist* 180(2): 200-210.

Fisher R (2005) Swimming speeds of larval coral reef fishes: impacts on self-recruitment and dispersal. *Marine Ecology Progress Series* 285: 223-232.

Fogarty MJ, Sissenwine MP, Cohen EB (1991) Recruitment variability and the dynamics of exploited marine populations. *Trends in Ecology and Evolution* 6: 241-246.

Funk WC, McKay JK, Hohenlohe PA, Allendorf FW (2012) Harnessing genomics for delineating conservation units. *Trends in Ecology and Evolution* 27: 489-496.

Gagliano M, McCormick MI, Meekan MG (2007) survival against the odds: ontogenetic changes in selective pressure mediate growth-mortality trade-offs in a marine fish. *Proceedings of the Royal Society B: Biological Sciences* 274: 1575-1582.

Gaines S, Brown S, Roughgarden J (1985) Spatial variation in larval concentrations as a cause of spatial variation in settlement for the barnacle, *Balanus glandula* . *Oecologia* 67: 267-272.

Gaines SD, Gaylord B, Gerber AHR, Hastings A, Kinlan BP (2007) Connecting places: the ecological consequences of dispersal in the sea. *Oceanography* 20: 90-99.

Gaines SD, Bertness MD (1992) Dispersal of juveniles and variable recruitment in sessile marine species. *Nature* 360: 579-580.

Galarza JA, Carreras-Carbonell JC, Macpherson E, Pascual M, Roques S, Turner GF, Rico C (2009) The influence of oceanographic fronts and early-life-history traits on connectivity among littoral fish species. *Proceedings of the National Academy of Sciences of USA* 106: 1473-1478.

Grantham BA, Eckert GL, Shanks AL (2003) Dispersal potential of marine invertebrates in diverse habitats. *Ecological Applications* 13: 108-116.

Hansson L (1991) Dispersal and connectivity in metapopulations. *Biological Journal of the Linnean Society* 42: 89-103.

Hastings A, Harrison S (1994) Metapopulation dynamics and genetics. *Annual Review of Ecology and Systematics* 25(1): 167-188.

Hawkins SJ, Côrte-Real HBSM, Martins HR, Santos RS, Martins AMF (1990a) A note on the identity of *Patella* in the Azores. *Açoreana* (Suppl.): 167-173.

Hawkins SJ, Burnay LP, Neto AI, Da Cunha RT, Martins AMF (1990b) A description of the zonation patterns of molluscs and other important biota on the south coast of São Miguel, Azores. *Açoreana* (Suppl.): 21-38.

Hawkins SJ, Hartnoll RG, Kain JM, Norton TA (1992) Plant-animal interactions on hard substrata m the North-West Atlantic. In *Plant-Animal Interactions in the Marine Benthos*. John DM, Hawkins SJ, Price JH (Eds). The Systematics Association, Special Vol. 46. Clarendon Press, Oxford. pp. l-32.

Hawkins SJ, Côrte-Real HBSM, Pannacciulli FG, Weber LC, Bishop JDD (2000) Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. *Hydrobiologia* 440: 3-17.

Hawkins SJ, Bohn K, Sima DW, Ribeiro P, Faria J, Presa P, Pita A, Martins GM, Neto AI, Burrows MT, Genner MJ (2016) Fisheries stocks from an ecological perspective: Disentangling ecological connectivity from genetic interchange. *Fisheries Research* 179: 333-341.

Hawkins SJ, Hartnoll RG (1982) Settlement patterns of *Semibalanus balanoides* in the Isle of Man (1977-1981). *Journal of Experimental Marine Biology and Ecology* 62: 271-283.

Hawkins SJ, Hartnoll RG (1983) Grazing of intertidal algae by marine invertebrates. *Oceanography and Marine Biology: an Annual Review* 21: 195-282.

Hawkins SJ, Hiscock K (1983) Anomalies in the abundance of common eulittoral gastropods with planktonic larvae on Lundy island, Bristol Channel. *Journal of Molluscan Studies* 49: 86-88.

Hedgecock D (1986) Is gene flow from pelagic larval dispersal important in the adaptation and evolution of marine invertebrates?. *Bulletin of Marine Science* 39: 550-564.

Hjort J (1914) Fluctuations in the great fisheries of northern Europe viewed in the light of biological research. *Rapports et Procès-Verbaux Des Réunions Du Conseil International Pour l'Exploration de la Mer* 1: 5-38.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. *Science* 293: 629-638.

Jarne P, Lagoda PJL (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* 11: 424-429.

Jenkins SR, Coleman RA, Santina PD, Hawkins SJ, Burrows MT, Hartnoll RG (2005) Regional scale differences in the determinism of grazing effects in the rocky intertidal. *Marine Ecology Progress Series* 287: 77-86.

Jenkins DG, Brescacin CR, Duxbury CV, Elliott JA, Evans JA, Grablow KR, Hillegass M, Lyon BN, Metzger GA, Olandese ML, Pepe D, Silvers GA, Suresch HN, Thompson TN, Trexler CM, Williams GE, Williams NC, Williams SE (2007) Does size matter for dispersal distance?. *Global Ecology and Biogeography* 16: 415-425.

Jenkins SR (2005) Larval habitat selection, not larval supply, determines settlement patterns and adult distribution in two chthamalid barnacles. *Journal of Animal Ecology* 74: 893-904.

Jones GP, Millicich MJ, Emslie MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. *Nature* 402: 802-804.

Jonsson PR, Granhag L, Moschella P, Aberg P, Hawkins SJ, Thompson RC (2006) Interactions between wave action and grazing control the distribution of intertidal macroalgae. *Ecology* 87: 1169-1178.

Keough MJ, Downes BJ (1982) Recruitment of marine invertebrates: the role of active larval choices and early mortality. *Oecologia* 54: 348-352.

Knutsen H, Jorde PE, André C, Stenseth NC (2003) Fine-scaled geographical population structuring in a highly mobile marine species: the Atlantic cod. *Molecular Ecology* 12: 385-394.

Lagos NA, Castilla JC, Broitman BR (2008) Spatial environmental correlates of intertidal recruitment: a test using barnacles in northern Chile. *Ecological Monographs* 78: 245-261.

Lasker R (1981) Factors contributing to variable recruitment of the northern anchovy (*Engraulis rnordax*) in the California current: contrasting years, 1975 through 1978. *Rapports et Procès-Verbaux Des Réunions Du Conseil International Pour l'Exploration de la Mer* 178: 375-388.

Levin LA (2006) Recent progress in understanding larval dispersal: new directions and digressions. *Integrative and Comparative Biology* 46: 282-297.

Marshall DJ, Morgan SG (2011) Ecological and evolutionary consequences of linked life-history stages in the sea. *Current Biology* 21: R718-R725.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Borges CDG, Vale M, Ferraz RR, Martins HR, Santos RS, Hawkins SJ (2017) Exploitation promotes earlier sex change in a protandrous patellid limpet, *Patella aspera* Röding, 1798. *Ecology and Evolution* 7(10): 3616-3622.

Martins HR, Santos RS, Hawkins SJ (1987a) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins HR, Santos RS, Hawkins SJ (1987b) *Estudos sobre as lapas dos Açores: Exploração e avaliação. Relatório da XVII Semana das Pescas dos Açores*. Universidade dos Açores.

McClanahan TR, Kamukuru AT, Muthiga NA, Gilagabher M, Obura D (1996) Effect of sea urchin reductions on algae, coral and fish populations. *Conservation Biology* 10: 136-154.

Menge BA (2000) Top-down and bottom up community regulation in marine rocky intertidal habitats. *Journal of Experimental Marine Biology and Ecology* 250: 257-289.

Mora C, Sale PF (2002) Are populations of coral reef fish open or closed?. *Trends in Ecology and Evolution* 17: 422-428.

Morgan S (2001) The larval ecology of marine communities. In *Marine community ecology.* Bertness M, Gaines S, Hay M (Eds). Sinauer associates, Sunderland. pp. 159-181.

Morgan SG, Fisher JL, Miller SH, McAfee ST, Largier JL (2009) Nearshore larval retention in a region of strong upwelling and recruitment limitation. *Ecology* 90: 3489-3502.

Morton B, Britton JC, Martins AMF (1998) *Coastal ecology of the Azores*. Sociedade Afonso Chaves, Ponta Delgada.

Murphy HM, Warren-Myers FW, Jenkins GP, Hamer PA, Swearer SE (2014) Variability in size-selective mortality obscures the importance of larval traits to recruitment success in a temperate marine fish. *Oecologia* 175: 1201-1210.

Navarro PG, Ramírez R, Tuya F, Fernandez-Gil C, Sanchez-Jerez P, Haroun RJ (2005) Hierarchical analysis of spatial distribution patterns of patellid limpets in the Canary Islands. *Journal of Molluscan Studies* 71: 67-73.

Nielsen EE, Hemmer-Hansen J, Larsen PF, Bekkevold D (2009) Population genomics of marine fishes: identifying adaptive variation in space and time. *Molecular Ecology* 18: 3128-3150.

Orensanz JM, Armstrong J, Armstrong D, Hilborn R (1998) Crustacean resources are vulnerable to serial depletion - the multifaceted decline of crab and shrimp fisheries in the Greater Gulf of Alaska. *Reviews in Fish Biology and Fisheries* 8: 117-176.

Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* 25: 547-572.

Pineda J, Hare JA, Sponaugle S (2007) Larval dispersal and transport in the coastal ocean and consequences for population connectivity. *Oceanography* 20: 22-39.

Poore AGB, Campbell AH, Coleman RA, Edgar GJ, Jormalainen V, Reynolds PL, Sotka EE, Stachowicz JJ, Taylor RB, Vanderklift MA, Duffy JE (2012) Global patterns in the impact of marine herbivores on benthic primary producers. *Ecology Letters* 15: 912-922.

Ribeiro PA, Branco M, Hawkins SJ, Santos AM (2010) Recent changes in the distribution of a marine gastropod, *Patella rustica*, across the Iberian Atlantic coast did not result in diminished genetic diversity or increased connectivity. *Journal of Biogeography* 37(9): 1782-1796.

Roughgarden J, Gaines S, Possingham H (1988) Recruitment dynamics in complex life cycles. *Science* 241: 1460-1466.

Sá-Pinto A, Branco MS, Harris DJ, Alexandrino P (2005) Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. *Journal of Experimental Marine Biology and Ecology* 325: 95-110.

Sá-Pinto A, Branco A, Sayanda D, Alexandrino P (2008) Patterns of colonization, evolution and gene flow in species of the genus *Patella* in the Macaronesian Islands. *Molecular Ecology* 17: 519-532.

Salgueiro, P, Palmeirim JM, Ruedi M, Coelho MM (2008) Gene flow and population structure of the endemic Azorean bat (*Nyctalus azoreum*) based on microsatellites: implications for conservation. *Conservation Genetics* 9: 1163-1171.

Sanford E, Holzman SB, Haney RA, Rand DM, Bertness MD (2006) Larval tolerance, gene flow, and the northern geographic range limit of fiddler crabs. *Ecology* 87: 2882-2894.

Santos RS, Martins HR, Hawkins SJ (1990) *Relatório de estudos sobre o estado das populaces de lapas do Arquipélago dos Açores e da ilha da Madeira.* Relatório da X Semana das Pescas dos Açores. Universidade dos Açores. Horta.

Santos RS, Hawkins SJ, Monteiro LR, Alves M, Isidro EJ (1995) Marine research, resources and conservation in the Azores*. Aquatic Conservation: Marine and Freshwater Ecosystems* 5: 311-354.

Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. *Chromossoma* 109: 365-371.

Schlötterer C (2004) The evolution of molecular markers - just a matter of fashion?. *Nature Review*

Schmidt PS, Rand DM (2001) Adaptive maintenance of genetic polymorphism in an intertidal barnacle: habitat- and life-stage-specific survivorship of *mpi* genotypes. *Evolution* 55: 1336-1344.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9: 615-629.

Siegel DA, Kinlan BP, Gaylord B, Gaines SD (2003) Lagrangian descriptions of marine larval dispersion. *Marine Ecology Progress Series* 260: 83–96.

Siegel DA, Mitarai S, Costello CJ, Gaines SD, Kendall BE, Warner RR, Winter KB (2008) The stochastic nature of larval connectivity among nearshore marine populations. *Proceedings of the National Academy of Sciences of the USA* 105: 8974-8979.

Smith ADM, Brown CJ, Bulman CM, Fulton EA, Johnson P, Kaplan IC, Lozano-Montes H, Mackinson S, Marzloff M, Shannon LJ, Shin YJ, Tam J (2011) Impacts of fishing low-trophic level species on marine ecosystems. *Science* 333: 1147-1150.

Sponaugle S, Boulay JN, Rankin TL (2011) Growth- and size-selective mortality in pelagic larvae of a common reef fish. *Aquatic Biology* 13: 263-273.

Stephenson RL (1999) Stock complexity in fisheries management: A perspective of emerging issues related to population sub-units. *Fisheries Research* 43: 247-249.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution* 15: 199-203.

Swearer SE, Shima JS, Hellberg ME, Thorrold SR, Jones GP, Robertson DR, Morgan SG, Selkoe KA, Ruiz GM, Warner RR (2002) Evidence of self-recruitment in demersal marine populations. *Bulletin of Marine Science* 70: 251-271.

Teske PR, Papadopoulos I, Mmonwa KL, Matumba TG, McQuaid CD, Barker NP, Beheregaray LB (2011) Climate-driven genetic divergence of limpets with different life histories across a southeast African marine biogeographic disjunction: different processes, same outcome. *Molecular Ecology* 20: 5025-5041.

Thornhill DJ, Mahon AR, Norenburg JL, Halanych KM (2008) Open-ocean barriers to dispersal: a test case with the Antarctic Polar Front and the ribbon worm *Parborlasia corrugatus* (Nemertea: Lineidae). *Molecular Ecology* 17: 5104-5117.

Treml EA, Roberts JJ, Chao Y, Halpin PN, Possingham HP, Riginos C (2012) Reproductive output and duration of the pelagic larval stage determine seascape-wide connectivity of marine populations. *Integrative & Comparative Biology* 52(4): 525-537.

Turner TF, Wares JP, Gold JR (2002) Genetic effective size is three orders of magnitude smaller than adult census size in an abundant, estuarine-dependent marine fish (*Sciaenops ocellatus*). *Genetics* 162: 1329-1339.

Underwood AJ (1980) The effects of grazing by gastropods and physical factors on the upper limits of distribution of intertidal macroalgae. *Oecologia* 46: 201-213.

Underwood AJ, Fairweather PG (1989) Supply-side ecology and benthic marine assemblages. *Trends in Ecology and Evolution* 4: 16-20.

Uriz MJ, Maldonado M, Turon X, Marti R (1998) How do reproductive output, larval behaviour, and recruitment contribute to adult spatial patterns in Mediterranean encrusting sponges?. *Marine Ecology Progress Series* 167: 137-148.

Warner RR, Cowen RK (2002) Local retention of production in marine populations: Evidence, mechanisms, and consequences. *Bulletin of Marine Science* 70: 245-249.

Weber LI, Hawkins SJ (2002) Evolution of the limpet *Patella candei* d'Orbigny (Mollusca: Patellidae) in Atlantic archipelagos: human intervention and natural processes. *Biological Journal of the Linnean Society* 77: 341-353.

Weber LI, Hawkins SJ (2005) *Patella aspera* and *P. ulyssiponensis*: genetic evidence of speciation in the North-east Atlantic. *Marine Biology* 147: 153-162.

Weber LI, Hawkins SJ (2006) Allozymic differentiation among geographically distant populations of *Patella vulgata* (Mollusca, Patellogastropoda). *Hydrobiologia* 553: 267-275.

Weersing K, Toonen R (2009) Population genetics, larval dispersal, and connectivity in marine systems. *Marine Ecology Progress Series* 393: 1-12.

Wolf H, Verhagen R, Backeljau T (2000) Large scale population structure and gene flow in the planktonic developing periwinkle, *Littorina striata*, in Macaronesia (Mollusca: Gastropoda). *Journal of Experimental Marine Biology and Ecology* 246: 69-83.

Wood AR, Gardner JPA (2007) Small spatial scale population genetic structure in two limpet species endemic to the Kermadec Islands, New Zealand. *Marine Ecology Progress Series* 349: 159-170.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* 11: 1-16.

CHAPTER 2

A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos

ABSTRACT

1. The intertidal limpet *Patella candei* represents an important exploited resource in the Macaronesian archipelagos of Azores, Madeira and Canaries (NE Atlantic). Despite being considered endemic, the taxonomic status of *P. candei* throughout the region remains unclear.

2. The aim of this study was to develop novel microsatellite markers using next generation sequencing for the limpet *P. candei*.

3. Twelve novel loci were described and amplified in 103 individuals from two populations (Azores and Madeira) using three multiplex reactions. The number of alleles per locus ranged from 2 to 14 and global observed heterozygosity was 0.417. Genetic differentiation between samples was highly significant (F_{ST} = 0.427) as two main gene pools (K = 2) were identified using Bayesian approaches.

4. The present genetic tool can be useful to establish the genetic structuring and patterns of connectivity of *P. candei* in Macaronesia as well as to determine the number of extant subspecies within the *P. candei* species complex. Such data will provide a fundamental asset to define stocks and thus inform specific conservation strategies that foster the sustainable exploitation of the *P. candei* complex throughout Macaronesia archipelagos.

KEYWORDS: intertidal, archipelago, genetics, biogeography, invertebrates, fishing

Published as:

Faria J, Pita A, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Neto AI, Presa P (2016) A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 775-781.
DOI: 10.1002/aqc.2651

Introduction

The Macaronesian (NE Atlantic) archipelagos comprise the oceanic islands of Azores, Madeira, Canaries and Cape Verde and are separated from the Euro-African mainland by between 100 km and 1 900 km. Several patellid limpet species can be found on these islands and are viewed as key species for the structuring and functioning of rocky shore communities, since they graze on algae and control their abundance (Coleman *et al.* 2006; Martins *et al.* 2010). Limpets have long been collected for human consumption but the stocks in several islands have been over-exploited and face severe collapse. Limpet overfishing has also resulted in ecological changes to the intertidal community structure with important ecosystem-level impacts (Martins *et al.* 2008; Martins *et al.* 2010). The limpet species *Patella candei* (d'Orbigny 1840) inhabits rocky shores from mid-intertidal to 5 m depth in all Macaronesian archipelagos except Cape Verde (Côrte-Real *et al.* 1996) and like *P. aspera*, is highly exploited for human consumption (Hawkins *et al.* 2000; Faria *et al.* 2015). *Patella candei*, as a Macaronesian endemic limpet, is threatened nowadays by over-exploitation and habitat disruption. The taxonomy of the *P. candei* species complex has prompted considerable scientific debate in recent decades (Côrte-Real *et al.* 1996; Weber and Hawkins, 2002; Sá-Pinto *et al.* 2005). For instance, a comprehensive taxonomic review of the genus *Patella* has assigned a particular subspecies to a specific archipelago, i.e. *P. candei gomesii* found throughout the Azores, *P. candei ordinaria* in Madeira, *P. candei crenata* in the Canary Islands, and *P. candei candei* in the Selvagens Islands and Fuerteventura island (for revision see Christiaens 1973). Later work using allozymes showed that *P. candei* from the Azores differed from *P. candei* in Madeira and Canary Islands (Côrte-Real *et al.* 1996). Weber and Hawkins (2002) also used allozyme electrophoresis to study populations of *P. candei* from the Macaronesian archipelagos and found two distinct genetic groups, one comprising *P. candei gomesii* and *P. candei candei* and the other comprising *P. candei crenata* and *P. candei ordinaria*. The authors further suggest that these two groups should be considered as two subspecies of *P. candei*, based on intermediate phenotypes found in the Canary Islands and Selvagens. Additional work done by Sá-Pinto *et al.* (2005) using more modern mtDNA techniques did not fully support previous findings. In their study, two well-differentiated groups were always identified in the Macaronesian islands: one including *P. candei* from the Azores, Desertas and Madeira, and another one grouping *P. candei* from the Canaries and Selvagens together with *Patella lugubris* from Cape Verde. The taxonomic status of Macaronesian limpets thus remains unclear with much controversy as to whether *P. candei* from the different archipelagos should be given a specific status. Despite its opportunistic life history (gonochoristic species with external fertilization, a pelagic larval stage, a relatively continuous pattern of recruitment, and a fast growth rate) populations of *P. candei candei* are now virtually extinct in all but one of the Canary Islands (Núñez *et al.* 2003; Navarro *et al.* 2005) and therefore listed in the Catalogue of Endangered Species of the Canary Islands as well as in the Spanish National Catalogue of Endangered Species. Similarly, *P. candei* abundance has dramatically decreased over the last decades in the Azores (Martins *et al.* 2011) and there is evidence of recruitment failure on its smaller islands (Martins *et al.* 2008).

The aim of this study was to develop and test a novel multiplex microsatellite tool from the *P. candei* genome that can be used to establish the genetic structure of populations and their biogeographic distribution patterns across the Macaronesian archipelagos. Such a novel molecular tool can provide a fundamental asset to define stocks and thus inform conservation strategies that foster the sustainable exploitation of *P. candei* throughout Macaronesia.

Methods

A total of 103 individuals of *P. candei* were collected from Terceira (Azores, n = 53) and Madeira (Madeira, n = 50) that lie around 1150 km apart from each other (Fig. 1). Genomic DNA was isolated from muscle tissue using the E.Z.N.A. Mollusc DNA extraction kit. The quality and quantity of DNA extractions were assessed using a Nanodrop spectrophotometer (Thermo Scientific). A DNA mix from five individuals (> 10 µg mL^{-1}) from Azores was used to develop microsatellite-enriched libraries with the new generation pyrosequencing GS-FLX titanium technology (454 Life Sciences, Roche) and reagents (Malausa *et al.* 2011 for details) as implemented by Genoscreen (France). In essence, small digested fragments from genomic DNA were PCR amplified and enriched for microsatellite content by using magnetic streptavidin beads and labelled TG, TC, AAC, AAG, AGG, ACG, ACAT and ACTC repeat oligonucleotide probes. Subsequent enrichments were PCR amplified, and the sequences obtained were analysed using the bioinformatic QDD pipeline (Meglécz *et al.* 2010). Sequences containing microsatellite motifs were identified and several primer pairs were tested for each marker until satisfactory PCR amplifications were achieved.

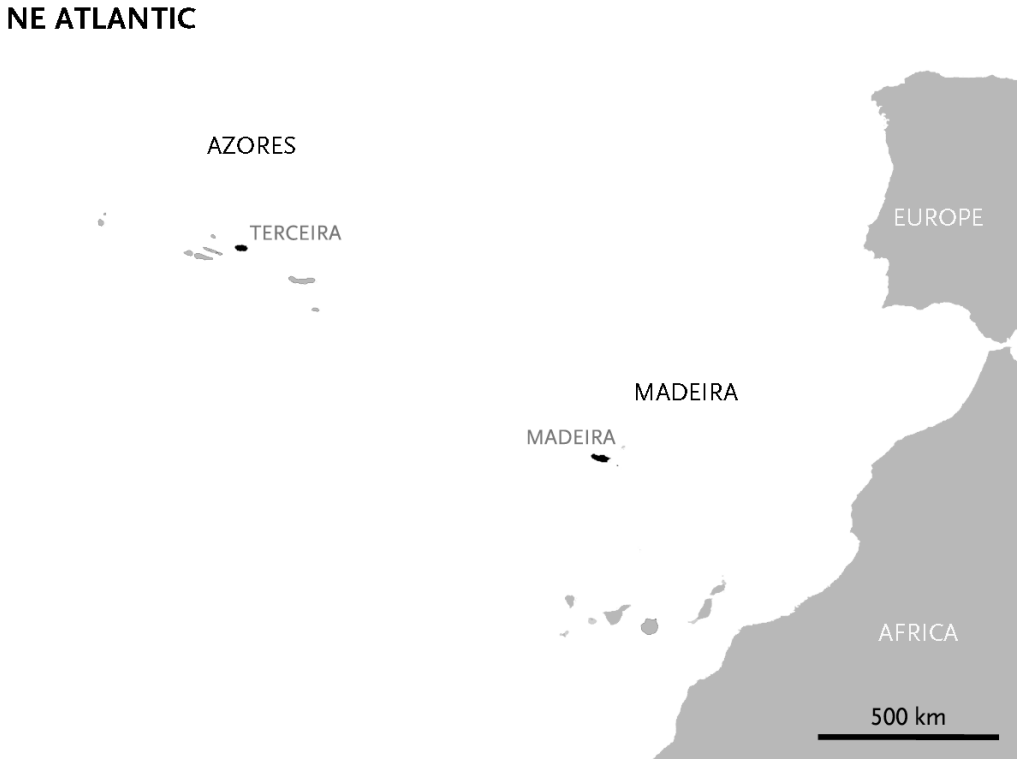


Figure 1. Location of sampling sites for *Patella candei* populations in the NE Atlantic.

Sixty-five primer pairs were selected for initial amplification testing in a subset of *P. candei* samples, following standard PCR protocol (QIAGEN). Polymorphic markers that amplified reliably were 5′ end-labelled in the forward primer using 6-FAM, VIC, NED or PET fluorophores (Applied Biosystems). In addition, a five-bp pig-tail sequence (GTTT) was added to the 5′ region of each reverse primer, to facilitate adenylation (Brownstein *et al.* 1996). Markers were then tested for multiplex compatibility. The number of markers included in each multiplex PCR was as high as possible, accounting for their allele size range, assigned dye label, and the avoidance of potential hairpin structures and primer dimers as indicated in AUTODIMER (Vallone and Butler 2004). MULTIPLEX MANAGER v.1.0 (Holleley and Geerts 2009) was used to combine all markers into the most efficient number of PCR reactions. After testing for suitable amplification of the selected multiplex, each setup was applied to genotype all *P. candei* samples. Multiplex PCR was carried out in 10 µL reactions containing ~30 ng DNA template, 1 × Qiagen™ Multiplex PCR Kit, 0.5 – 1.2 µmol L^{-1} of each primer and ddH_2O. Parameters for thermal cycling were as follows: 95 °C for 15 min, eight touchdown cycles (reduction of 0.5 °C per cycle) at 95 °C for 30 s, annealing temperature (T_A + 2 °C; Table 1) for 60 s, and 72°C for 40 s; 25 cycles at 95°C for 30 s, T_A for 60 s, and 72°C for 40 s; 10 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s. A final elongation was performed at 60 °C for 30 min. Genotyping was performed on an ABI 3730 (Applied Biosystems) automated DNA sequencer using an internal size standard (GeneScan™ 500Liz®, Applied Biosystems) for accurate sizing and GENEMAPPER™ v.4.2 (Applied Biosystems) was used for allele calling.

Table 1. Characteristics of 12 microsatellite loci developed from *P. candei* genome and casted in three multiplex PCR reactions.

Locus	Repeat motif	Primer Sequence (5'-3')	Multiplex (Fluorescent dye)/ T_A (°C)	Concentration (mM)	Size range (bp)	GenBank Accession n°
CAN18	$(AG)_9$	F: CCCATTTGTGCCCATTTCTA R: CCCTACGCACAGCGAATTAT	PcaMix1 (VIC)/ 60	1.2	162-194	KT879796
CAN25	$(AG)_{11}$	F: CCAAATCAGCGAATTTGAAC R: CGTGACTGTCAGAGCAGAGC	PcaMix1 (NED)/ 60	0.6	163-185	KP322707
CAN27	$(GA)_9$	F: CCCCAATGAATTCCAACAGA R: GGGTTCTGATTTTCAATGGGA	PcaMix1 (FAM)/ 60	0.7	181-193	KP322709
CAN53	$(CT)_7$	F: GTGATAGTGATGCGTGCG R: CCACGTATGACAGCCATCTC	PcaMix1 (PET)/ 60	0.8	129-143	KP322713
CAN9	$(TG)_{11}$	F: CCTTAAAGCGCTATTTCAGCA R: TCTCAGTGGTGGAGGAATCA	PcaMix2 (PET)/ 58	0.8	141-165	KT879797
CAN26	$(TC)_{91}$	F: CCCCTACCCGAAATGACCTA R: CAATGTGGATTTGAATTGACAAA	PcaMix2 (VIC)/ 58	0.5	186-190	KP322708
CAN32	$(AG)_8$	F: TGCTCGTGGCTGTGATTTAG R: ACGTAGGGAGTGCATCGTTT	PcaMix2 (NED)/ 58	0.9	225-252	KP322710
CAN40	$(TC)_7$	F: GTCATTTCAGGGCAACGATT R: TCCCTAGACAGTTGCAAATCA	PcaMix2 (FAM)/ 58	1.2	289-351	KP322712
CAN60	$(CT)_6$	F: AACACCAGGACAAGTGAACG R: GTGAGTGACCGCATTAGCTC	PcaMix3 (NED)/ 58	0.5	117-127	KP322715
CAN23	$(GA)_8$	F: AGATATCCGCAATGCACTCA R: GTCGAGTGGACGTTAAACCC	PcaMix3 (PET)/ 58	1.2	149-173	KP322706
CAN33	$(CT)_9$	F: GTCCAACGTCATGGCTTTTC R: ATGGTCCCATGCAATGCT	PcaMix3 (VIC)/ 58	0.7	231-235	KP322711
CAN56	$(TG)_7$	F: TATCGTCATCGCCTTTGACT R: GGGCATGCACCAGAAATAAT	PcaMix3 (FAM)/ 58	0.6	156-168	KP322714

F = forward primer sequence; R = reverse primer sequence; T_A = annealing temperature

Standard genetic diversity estimates (observed and expected heterozygosities – H_O and H_E) and tests for linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) were calculated using GENEPOP v.4.2 (Raymond and Rousset 1995). The presence of null alleles was assessed at a 95% confidence interval using MICRO-CHECKER v.2.2.3 (van Oosterhout *et al.* 2004). Null allele frequencies were estimated for each locus and population with FREENA (Chapuis and Estoup 2007). Genetic differentiation between samples (F_{ST}) was calculated using with ARLEQUIN v.3.5.1.3 (Excoffier and Lischer 2010) following Weir and Cockerham (1984) and FREENA, which uses Weir (1996) calculations and the so-called ENA correction method for the positive bias on F_{ST} induced by the presence of null alleles (Chapuis and Estoup 2007). Population structure was assessed using the Bayesian model-based clustering approach implemented in STRUCTURE v.2.3.4 (Pritchard *et al.* 2000) and individuals were assigned to a given cluster without using any prior information about their origin. Correlated allele frequencies and the admixture model were assumed. Ten independent runs were made for K = 1 – 2 with each run consisting of a burn-in of 10^5 Markov-chain Monte Carlo steps, followed by 5×10^5 iteration steps. Selection of the most likely number of genetic clusters (K) was based on Evanno *et al.* (2005) ΔK method. For comparison purposes, genetic differentiation between samples was estimated either on all the loci set, or on a subset of loci after removing those bearing putative null alleles. Reassignment of significance levels for multiple tests comparison was performed using the sequential Bonferroni correction (Rice 1989).

Results

The GS-FLX sequencing platform produced 30 683 individual sequences, of which 1 799 contained microsatellite motifs. Primer pairs were designed for 107 potential markers, where 88% of them were dinucleotides, 10% trinucleotides and 2% tetranucleotides. Several primer pairs out of the 65 selected for initial amplifications were discarded either because they showed tri-allelic patterns, exhibited artifact bands interfering with allele calling, and/or were monomorphic for all samples screened. Twelve primer pairs that showed clean polymorphisms were combined in three optimized multiplex reactions and assayed in all samples (Table 1).

Preliminary analyses showed that populations are well differentiated and therefore genetic diversity indices were calculated for each population separately. All loci amplified in the Azorean sample. In contrast, five loci failed to amplify in individuals from Madeira (Table 2). Loci CAN9, CAN26 and CAN33 did not amplify at all and two additional loci failed to amplify in more than 30% of the Madeira sample size. Since microsatellite markers were developed from Azorean *P. candei* individuals, the amplification failure of five loci in Madeira sample, suggests that the genetic differentiation between samples is very significant.

The number of alleles per locus ranged from 2 to 14 in Terceira (Azores) and showed average observed (H_O) and expected (H_E) heterozygosity of 0.359 and 0.436, respectively (Table 2). The number of alleles in the Madeira sample ranged from 2 to 12, and observed (H_O) and expected (H_E) heterozygosity were 0.289 and 0.479, respectively (Table 2).

Table 2. Genetic variation for twelve microsatellites in two populations of *Patella candei* from the Macaronesia archipelagos of Azores and Madeira.

	Terceira (AZORES)						Madeira (MADEIRA)					
Locus	N_A	H_O	H_E	F_{IS}	Null	Missing	N_A	H_O	H_E	F_{IS}	Null	Missing
CAN18	14	0.462	0.853	0.459*	**0.21**	2	5	0.242	0.673	0.640*	**0.26**	28
CAN25	7	0.481	0.688	0.302*	**0.13**	2	5	0.133	0.464	0.713*	**0.21**	70
CAN27	5	0.472	0.529	0.108	0.06	0	3	0.449	0.488	0.080	0.02	2
CAN53	5	0.302	0.313	0.036	0.00	0	6	0.633	0.563	-0.124	0.00	2
CAN9	10	0.774	0.783	0.012	0.02	0	NA	NA	NA	NA	NA	100
CAN26	3	0.510	0.640	0.203	0.08	8	NA	NA	NA	NA	NA	100
CAN32	4	0.327	0.347	0.059	0.03	2	12	0.723	0.850	0.149	0.04	6
CAN40	9	0.327	0.425	0.230*	**0.07**	2	7	0.148	0.673	0.780*	**0.31**	46
CAN60	2	0.113	0.141	0.198	0.04	0	5	0.104	0.479	0.783*	**0.27**	4
CAN23	4	0.481	0.429	-0.119	0.00	2	3	0.143	0.135	-0.057	0.00	2
CAN33	3	0.019	0.057	0.665	**0.09**	2	NA	NA	NA	NA	NA	100
CAN56	3	0.038	0.038	-0.005	0.00	2	2	0.024	0.024	NA	0.00	18

N_A number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient, *Null* frequency of null alleles, *Missing* percentage of missing data; *Significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction for multiple comparisons ($P < 0.05$). Bold values indicate null allele detection by MICROCHECKER.

There was no evidence of linkage disequilibrium among pairs of loci and three loci in Terceira and four loci in Madeira deviated significantly from HWE (Table 2). Probable causes for such deficit are non-random mating, population subdivision (Wahlund effect) or the presence of null alleles (Allendorf and Luikart 2007). It is noteworthy that all loci causing departures from HWE showed a high putative frequency of null alleles as indicated by MICRO-CHECKER and FREENA (Table 2). Again, null alleles were more often found in the genotypic distributions of Madeira, suggesting high amplification mispriming due to its genetic divergence from the Azorean sample.

Genetic differentiation between samples was very high and significant ($F_{ST\_ARLEQUIN} = 0.427$, $P < 0.001$). F_{ST} estimates obtained by excluding null alleles were slightly lower (F_{ST_FREENA} corrected for null alleles = 0.336; 95% CI (0.139 – 0.486) than the ones obtained when null alleles were ignored ($F_{ST\_FREENA} = 0.359$; 95% CI (0.159 – 0.515)). In this case, the occurrence of null alleles seems to have little impact on estimates of population differentiation. Strong subpopulation structure was also detected using Bayesian inferences, with the number K = 2 being chosen by the ad hoc statistic ΔK as the most probable number of gene pools present in the sample system, a result that agrees with the high F_{ST} divergence level observed between samples. Similar results were obtained when restricting the differentiation analysis to the loci set that did not exhibit null alleles (data not shown).

Discussion

Present results performed with a new genetic tool on two *P. candei* samples from Macaronesia provide evidence of a large demographic uncoupling among geographically distant limpet populations. Such a phylogeographic break is likely due to limited larval connectivity among archipelagos since adult patellid limpets are broadcast benthic spawners and the planktonic larval stage susceptible to

passive dispersal is believed to range from a few days up to 27 days (Ribeiro 2008; Nakano and Sasaki 2011). While many other factors can be involved (Cowen and Sponaugle 2009 for review), the putative limited dispersal ability of patellids, and the relationship between their life-history characteristics and ocean circulation processes probably contributed to the genetic differentiation observed between archipelagos. In the context of fisheries management, population discrimination from seemingly uniform stocks is paramount to correctly designate discrete management units for conservation. Any conservation strategy aimed at the sustainable exploitation of *P. candei* throughout Macaronesia should be archipelago-specific, and consider aspects such as local population demography (e.g. limpet densities) and region-specific life-history traits (e.g. time of reproduction and recruitment).

This study provides important insights into both the potential use of microsatellites in addressing the genetic population structure of *P. candei* throughout Macaronesian archipelagos and the micro-evolutionary processes that operated on the phylogeography of limpet populations across these isolated oceanic islands. In particular, it will be important to understand the scale and direction of connectivity among islands within each archipelago and to address inter-island population source–sink dynamics. For example, population genetic studies of the endemic Hawaiian limpets have shown significant subpopulation structures in three species of *Cellana* spp. Those results prompted their incorporation into management plans to deter previous poor conservation views of a single management unit (Bird *et al.* 2007).

This novel multiplex microsatellite tool can be used to underpin the delimitation of suitable management areas by identifying barriers to dispersal within each archipelago, and provide the population genetic basis for the formulation of stock-specific conservation strategies for the sustainable exploitation of *P. candei* throughout Macaronesia. Genetic information can also be useful to assess the effectiveness of current marine protected areas acting as larvae source to adjacent populations. It is clear that these endemic species require careful management if extinction is to be avoided as appears to have happened with *P. candei candei* in much of its range in the Canary archipelago.

Acknowledgments

This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects PTDC/BIA-BIC/ 115837/2009 and PEst-C/MAR/LA0015/2013, by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020 and by cE3c funding (Ref: UID/BIA/00329/2013). It was also partly supported by CIRN (Centro de Investigação de Recursos Naturais, University of the Azores), and CIIMAR (Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal). JF was funded by a PhD grant M3.1.2/ F/021/2011 by the Regional Government of the

Azores. GMM and PR were supported by post-doctoral grants awarded by FCT, Portugal (SFRH/ BDP/63040/2009 and SFRH/BPD/69232/2010 respectively).

References

Allendorf FW, Luikart G (2007) *Conservation and the genetics of populations*. Blackwell Publishing, London.

Bird CE, Holland BS, Bowen BW, Toonen RJ (2007) Contrasting phylogeography in three endemic Hawaiian limpets (*Cellana* spp.) with similar life histories. *Molecular Ecology* 16: 3173–3186.

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques* 20: 1004-1010.

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621-631.

Christiaens J (1973) Révision du genre *Patella* (Mollusca, Gastropoda). *Bulletin du Muséum National D'histoire Naturelle* 182: 1305-1392.

Coleman RA, Underwood AJ, Benedetti-Cecchi, Aberg P, Arenas F, Arrontes J, Castro J, Hartnoll RG, Jenkins SR, Paula J, Santina PD, Hawkins SJ (2006) A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia* 147: 556-564.

Côrte-Real HBSM, Hawkins SJ, Thorpe JP (1996) Population differentiation and taxonomic status of the exploited limpet *Patella candei* in the Macaronesian Islands (Azores, Madeira, Canaries). *Marine Biology* 125: 141-152.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review of Marine Science* 1: 443-466.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611-2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46(1): 96-97.

Hawkins SJ, Côrte-Real HBSM, Pannacciulli FG, Weber LC, Bishop JDD (2000) Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. *Hydrobiologia* 440: 3-17.

Holleley CE, Geerts PG (2009) Multiplex Manager 1.0: a crossplatform computer program that plans and optimizes multiplex PCR. *Biotechniques* 46: 511-517.

Malausa T, Gilles A, Meglécz E, Blanquart H, Duthoy S, Costedoat C, Dubut V, Pech N, Castagnone-Sereno P, Délye C, Feau N, Frey P, Gaulthier P, Guillemaud T, Hazard L, Le Corne V, Lung-Escarmant B, Malé PJ, Ferreira, Martin JF (2011) High-throughput microsatellite isolation through 454 GS-FLX titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11: 638-644.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Thompson RC, Neto AI, Hawkins SJ, Jenkins SR (2010) Exploitation of intertidal grazers as a driver of community divergence. *Journal of Applied Ecology* 47: 1282-1289.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Medeiros AR, Thompson RC (2011) Illegal harvesting affects the success of fishing closure areas. *Journal of the Marine Biological Association UK* 91: 929-937.

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin JF (2010) QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects *Bioinformatics* 26: 403-404.

Nakano T, Sasaki T (2011) Recent advances in molecular phylogeny, systematics and evolution of patellogastropod limpets. *Journal of Molluscan Studies* 77: 203–217.

Navarro PG, Ramírez R, Tuya F, Fernandez-Gil C, Sanchez-Jerez P, Haroun RJ (2005) Hierarchical analysis of spatial distribution patterns of patellid limpets in the Canary Islands. *Journal of Molluscan Studies* 71: 67-73.

Núñez J, Brito MC, Riera R, Docoito JR, Monterroso O (2003) Distribución actual de las poblaciones de *Patella candei* d'Orbigny, 1840 (Mollusca, Gastropoda) en las islas Canarias - Una especie en peligro de extinción. *Boletin Instituto Espanol de Oceanografia* 19: 371-377.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Raymond M, Rousset F (1995) GENEPOP (version 1.2) - population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Rice WR (1989) Analyzing tables and statistical tests. *Evolution* 43: 223-225.

Sá-Pinto A, Branco MS, Harris DJ, Alexandrino P (2005) Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. *Journal of Experimental Marine Biology and Ecology* 325: 95-110.

Vallone PM, Butler JM (2004) AutoDimer: a screening tool for primer-dimer and hairpin structures. *Biotechniques* 37: 226-231.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535-538.

Weber LI, Hawkins SJ (2002) Evolution of the limpet *Patella candei* d'Orbigny (Mollusca: Patellidae) in Atlantic archipelagos: human intervention and natural processes. *Biological Journal of the Linnean Society* 77: 341-353.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.

Weir BS (1996) *Genetic Data Analysis II*. Sinauer Associates: Sunderland, MA.

CHAPTER 3

A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798)

Published as:

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46(1): 96-97. DOI: 10.1111/age.12243

Background

Patellid limpets are ecologically important keystone grazers having a long history of overexploitation in the Macaronesian Archipelagos (NE Atlantic islands), where some species, such as *Patella aspera*, are under serious risk (Hawkins *et al.* 2000; Martins *et al.* 2008). *Patella aspera* is a protandric sequential hermaphrodite species with external fertilization, in which individuals start as males but may undergo a sex reversal with age (Martins *et al.* 1987). Hence, exploitation tends to focus on the larger females in the population as larger limpets (predominantly females) are selectively removed. Despite conservation legislation in Canaries, Madeira and Azores, limpets are under severe pressure and few individuals survive long enough to become females, a phenomenon that severely restricts the effective population size (Waples 2006). New conservation actions for the protection and sustainable use of limpets in Macaronesian archipelagos are urgently needed and should be based on a multidisciplinary framework based on knowledge of the population dynamics and connectivity of this species.

Samples, genetic analysis and results

A total of 309 microsatellite loci were isolated from the *P. aspera* genome using a 454 sequencing platform, and three multiplex sets comprising 17 loci for rapid population genetic analyses were developed in this species (see Supplementary Material for methodological details; Table S1). Samples from three populations of *P. aspera* (n = 127) from the Macaronesian islands (two from the archipelago of Azores and one from the Canaries) were fully genotyped using capillary electrophoresis. Genetic diversity and population differentiation estimates were assessed. All loci were polymorphic, and a large deficit in heterozygosity was commonplace across markers and samples (Table 1; Table S2). Such a deficit probably arose as a consequence of complex population processes resulting from overexploitation as well as from the usual null allele impact in molluscan microsatellites. Nonetheless, no proper inferences on the causality of this phenomenon can be made until assessed by large-scale population genotyping studies.

Table 1. Mean genetic diversity estimates based on 17 microsatellite loci for three *Patella aspera* populations from the Macaronesian islands of S. Miguel (Azores, SMI_1 and SMI_2) and Gran Canaria (Canaries, CAN).

	n	N_A (±SE)	H_O (±SE)	H_E (±SE)	F_{IS} (±SE)
SMI_1	48	10.1 (1.5)	0.393 (0.050)	0.718 (0.042)	0.463* (0.056)
SMI_2	49	10.5 (1.4)	0.381 (0.051)	0.732 (0.039)	0.497* (0.062)
CAN	30	8.2 (0.9)	0.278 (0.061)	0.669 (0.052)	0.607* (0.078)
All	127	9.6 (0.7)	0.351 (0.032)	0.707 (0.026)	0.514* (0.057)

n, number of individuals; N_A, number of alleles per sample; H_O, observed heterozygosity; H_E, unbiased expected heterozygosity; F_{IS}, inbreeding coefficient; SE, standard error. * indicates a significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction.

Pairwise comparisons of diversity among the three populations analysed showed a significant F_{ST} distance in 12 of the 17 markers analysed (Table S2), averaging $F_{ST} = 0.047$ ($P < 0.001$). Noteworthy, hierarchical AMOVAs showed that such interpopulation genetic distance was caused by the divergence between each of the Azorean samples and the Canarian sample ($F_{ST} = 0.074$, $P < 0.001$), but not between the Azorean samples ($F_{ST} = 0.017$, $P > 0.01$) (Table S3).

Comments

Despite the low number of samples analysed, present data suggest that the divergence found between archipelagos amounts to that expected between allopatric species rather than among conspecific populations. Next steps include the study of the genetic population structure of *P. aspera* throughout its distribution range so that future efforts can focus on identifying scales of connectivity and hence stock integrity across the Macaronesian Archipelagos. These novel tools can be useful to inform management of fisheries and preserve endemic limpet stocks that have been severely depleted in Macaronesia.

Acknowledgements

This research was developed under the Fundação para a Ciência e Tecnologia (FCT) project PTDC/BIA-BIC/115837/2009 and supported by the European Regional Development Fund (ERDF) through the COMPETE—Operational Competitiveness Program and national funds through FCT under the projects PTDC/BIA- BIC/115837/2009 and PEst-C/MAR/LA0015/2011. JF was funded by Fundo Regional para a Ciência (FRC) project M3.1.2/F/021/2011. GMM and PAR were supported by postdoctoral grants awarded by FCT (SFRH/ BDP/63040/2009 and SFRH/BPD/69232/2010 respectively.

References

Hawkins SJ, Côrte-Real HBSM, Pannacciulli FG, Weber LC, Bishop JDD (2000) Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. *Hydrobiologia* 440: 3-17.

Martins HR, Santos RS, Hawkins SJ (1987) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Waples RS (2006) A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. *Conservation Genetics* 7: 167-184.

SUPPLEMENTARY MATERIAL

Methods

Genomic DNA was extracted from foot tissue samples preserved in 96% ethanol using the E.Z.N.A. Mollusc DNA extraction kit (Omega Bio-tek). A DNA admixture from ten individuals (10 µg ml^{-1}) was used to build an enriched microsatellite library (Genoscreen, France). Fragments from genomic DNA were enriched in single sequence repeats using magnetic streptavidin beads and labelled oligonucleotide probes, i.e. $(TG)_n$, $(TC)_n$, $(AAC)_n$, $(AAG)_n$, $(AGG)_n$, $(ACG)_n$, $(ACAT)_n$ and $(ACTC)_n$. High-throughput microsatellite isolation was made upon 454 GS-FLX® Titanium (Roche) pyrosequencing on the enriched library. A total of 28 992 raw sequences were produced and titrated with QDD software (Meglécz *et al.* 2010) to recover 4 754 sequences containing microsatellites. Validation of primer pairs was achieved on 309 reads comprising 89% dinucleotides, 9% trinucleotides and 2.0% tetranucleotides. Forty pairs of primers (25 di-, 14 tri-, and 1 tetranucleotides) with high and close melting temperature, compatible allelic ranges and no misspriming were tested individually and PCR gradient conditions were assayed to check for their polymorphism across 10 trial samples. Twenty-three markers were discarded due to artifactual PCR bands or monomorphism. A final set of 17 optimally-amplified polymorphic loci showing amplicon size range of 110 bp - 353 bp was selected and their forward primers were fluorescently labelled with NED, VIC, PET, or 6‑FAM (Applied Biosystems, Life Technologies) and a GTTT sequence "pig-tail" tag was added to the 5' end of all reverse primers (Brownstein *et al.* 1996). Marker amplification was carried out in a MyCycler™ thermal cycler (BioRad) using a touchdown PCR protocol on a final volume of 10 µL containing 10 ng DNA template, 1 Qiagen™ Multiplex PCR Kit 0.4 - 1.2 M of each primer and ddH_2O. The PCR routine consisted of an initial denaturation step at 95°C for 15 min; 5 cycles of touchdown amplification (denaturation at 94°C for 30 sec, annealing temperature (T_A) for 1 min descending by 0.2°C intervals to the optimal T_A (Table S1), plus extension at 72°C for 40 sec; 22 cycles at 94°C for 30 sec, optimal T_A (Table S1) for 1 min, and extension at 72°C for 40 sec; 10 cycles at 94°C for 30 sec, 53°C for 50 sec, 72°C for 45 sec; and a final extension step at 60°C for 30 min. The 17 markers calibrated were combined in three multiplexed PCR sets for a more rapid and cost-efficient multilocus genotyping using AUTODIMER (Vallone and Butler 2004) (Table S1).

Multiplex PCR followed a similar protocol as described above, whereas cycle adjustments were only applied to the touchdown amplification in all mixes and T_A was lowered by 0.5°C on each consecutive cycle up to the optimal T_A. Amplified fragments were electrophoresed in an ABI 3730 automatic DNA sequencer (Applied Biosystems) using the internal size standard GeneScan™ 500Liz® (Applied Biosystems) and genotypes were scored using GENEMAPPER™ v.4.2 (Applied Biosystems). The number of alleles per locus and sample, observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficients (F_{IS}), deviations from the Hardy – Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated in GenAlEx v.6.5 (Peakall and Smouse 2012). Allelic richness (A_R) was estimated after the rarefaction method implemented in ADZE v.1.0 (Szpiech *et al.* 2008). MICRO-

CHECKER v. 2.2.3 was used to assess the presence of scoring errors and putative null alleles (van Oosterhout *et al.* 2004). Global and single-locus F_{ST} estimates were calculated with ARLEQUIN v.3.5.1.3 (Excoffier and Lischer 2010). Hierarchical partition of the genetic diversity within and between archipelagos was performed with an analysis of molecular variance (AMOVA) as implemented in ARLEQUIN. Multiple statistical tests were nominally adjusted with the sequential Bonferroni method (Rice 1989).

References

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques* 20: 1004-1010.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin JF (2010) QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects *Bioinformatics* 26: 403-404.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537-2539.

Rice WR (1989) Analyzing tables and statistical tests. *Evolution* 43: 223-225.

Szpiech ZA, Jakobsson M, Rosenberg NA (2008) ADZE: a rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* 27: 2498-2504.

Vallone PM, Butler JM (2004) AutoDimer: a screening tool for primer-dimer and hairpin structures. *Biotechniques* 37: 226-231.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535-538.

Table S1. Characteristics of seventeen microsatellite loci developed for *Patella aspera* and co-amplified in three multiplex reactions (PasMix I, II, III). F = forward primer sequence; R = reverse primer sequence; F/R = primer concentration; T_A = annealing temperature; N_A = number of alleles observed across samples.

Locus	Repeat motif	Primer sequence (5'-3')	F/R (mM)	Multiplex name (dye)	T_A (°C)	N_A	Size range (bp)	GenBank accession nº
ASP2F	$(AAG)_n$	F: CGTACTTCAATTGGCGAAGC R: CGATCTAGCAACCCCTGACT	1	PasMixI (NED)	60	11	110-146	KM594504
ASP3F	$(TCT)_n$	F: TTTCTGTCATGTGACTTTCTATCCTC R: TCTATGATGCCACACCTCCTT	0.8	PasMixI (VIC)		9	110-134	KM594514
ASP21F	$(AG)_n$	F: GTGCATAAAATTTGGTTGCG R: GAACCTGCAATAAGGAGATTTACA	0.5	PasMixI (PET)		12	168-188	KM594509
ASP23F	$(TCA)_n$	F: TATGAACCGCCCCTTTAAGA R: GCTGAGGGTCATGGTCATGT	1.2	PasMixI (FAM)		17	165-216	KM594518
ASP33F	$(AG)_n$	F: GGTTCTACGTAAGACCGTCTGG R: TCTTCAAATGAGGACTAAGCAGTT	1	PasMixI (FAM)		16	235-275	KM594516
ASP34F	$(GA)_n$	F: AGATGTCCCATCTGAGGTGC R: TACCCACCCGAACGACTATG	1.2	PasMixI (NED)		8	245-275	KM594515
ASP15F	$(TTC)_n$	F: ACAGTCACGAACAGGGTATGTT R: CCTCCCACAAGAAAGACTCCT	0.5	PasMixII (PET)	58	36	156-222	KM594512
ASP24F	$(GA)_n$	F: ATGTTCAAGCGATTGGAAGG R: GCAGAACATTGTGACCCAAA	1	PasMixII (VIC)		25	160-214	KM594507
ASP29F	$(CT)_n$	F: TCTCCGTGTACTCCGGTTTC R: GCCTGTCTGTTTTACACGCG	1	PasMixII (FAM)		18	211-248	KM594505
ASP36F	$(GA)_n$	F: ACCCTTTTGTGTGATCAGGG R: TTGTTTGTAGTGGATGTTGAAGC	0.5	PasMixII (NED)		16	288-344	KM594513
ASP38F	$(AG)_n$	F: GAAGTTTATATCACTCAGGGCCTA R: AGTCTAGAGTGCCCGCCTT	1	PasMixII (FAM)		12	302-352	KM594506
ASP39F	$(ACC)_n$	F: TGTTGGATATAGAGCGTGTTTTCA R: TTCACCTAGGGGAGGGATAGA	1	PasMixII (PET)		8	315-342	KM594517
ASP7F	$(ATC)_n$	F: CTGTCTTTCTCGTCTCACTCTCA R: GGTTGTGGACGTTGAACTGAT	0.4	PasMixIII (PET)	58	5	133-148	KM594520
ASP17F	$(AG)_n$	F: ATAAATAAATGTACAACCATTGACACA R: TACGGTTGTACGTGACAAGGA	0.6	PasMixIII (VIC)		9	148-168	KM594510
ASP26F	$(AG)_9$	F: ATTGGTGCACACCCACAATTA R: ATTGAGTCACCGGCGTAGTT	0.4	PasMixIII (FAM)		11	194-232	KM594519
ASP27F	$(TC)_{10}$	F: TTTTCTCAGGGTACTCCGGTT R: GGCATAATGGCAGGGTGAAT	0.4	PasMixIII (NED)		12	195-225	KM594508
ASP40F	$(CA)_8$	F: CAATTTCATTGACGCAAAGC R: CCCCACCAAATTGTATGAGC	0.4	PasMixIII (VIC)		15	311-353	KM594511

Table S2. Gene diversity of 17 microsatellite loci in three *Patella aspera* samples from the Macaronesian islands of S. Miguel (Azores, SMI_1, n = 48 and SMI_2, n = 49) and Gran Canaria (Canaries, n = 30). N_A = number of alleles per sample; $A_R(g)$ = allelic richness (g accounts for the maximum standardized sample size, i.e., twice the number of genotypes); H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; F_{IS} = inbreeding coefficient; F_{ST} = fixation index; * indicates a significant departure from Hardy-Weinberg equilibrium after sequential Bonferroni correction (* P < 0.01; ** P < 0.001, ns: non significant).

	N_A			A_R (4)			H_O / H_E			F_{IS}			F_{ST}
Locus	SMI_1	SMI_2	CAN	SMI_1	SMI2	CAN	SMI_1	SMI_2	CAN	SMI_1	SMI_2	CAN	All samples
ASP2	7	7	8	2.8	2.9	3.2	0.553/ 0.755	0.630/ 0.796	0.050/ 0.866	0.267^{ns}	0.208^{ns}	0.942**	0.001^{ns}
ASP3	7	8	5	2.3	2.5	2.3	0.213/ 0.574	0.273/ 0.630	0.000/ 0.618	0.629**	0.567**	1.000**	0.333**
ASP21	7	9	9	2.8	2.8	2.5	0.674/ 0.764	0.489/ 0.739	0.550/ 0.667	0.117^{ns}	0.338**	0.176^{ns}	0.079*
ASP23	13	15	12	3.1	3.3	3.3	0.723/ 0.830	0.848/ 0.867	0.643/ 0.879	0.129^{ns}	0.022^{ns}	0.269**	0.023*
ASP33	10	10	13	2.8	2.9	3.1	0.348/ 0.753	0.500/ 0.777	0.524/ 0.813	0.538**	0.356**	0.356**	0.022^{ns}
ASP34	5	7	6	2.5	2.7	2.5	0.233/ 0.678	0.279/ 0.736	0.160/ 0.662	0.657**	0.621**	0.758**	0.046^{ns}
ASP15	29	26	11	3.8	3.8	3.6	0.422/ 0.968	0.467/ 0.963	0.200/ 0.952	0.564**	0.516**	0.790**	0.026*
ASP24	23	21	16	3.6	3.6	3.6	0.674/ 0.935	0.565/ 0.936	0.700/ 0.929	0.280**	0.396**	0.246^{ns}	0.028*
ASP29	10	14	9	2.9	3.3	2.6	0.333/ 0.790	0.303/ 0.880	0.300/ 0.680	0.578**	0.656**	0.559**	0.062*
ASP36	13	9	8	3.1	2.8	2.8	0.256/ 0.841	0.195/ 0.753	0.389/ 0.776	0.695**	0.741**	0.499**	0.039*
ASP38	9	8	8	2.8	2.7	3.0	0.217/ 0.767	0.239/ 0.726	0.308/ 0.827	0.716**	0.679**	0.628**	0.185**
ASP39	5	7	1	1.3	1.9	1.0	0.087/ 0.167	0.298/ 0.436	0.000/ 0.000	0.479**	0.317*	NA	0.060*
ASP7	4	2	2	2.0	1.6	2.0	0.114/ 0.544	0.000/ 0.312	0.000/ 1.000	0.791**	1.000**	1.000	0.265*
ASP17	8	7	7	2.8	2.9	2.2	0.378/ 0.761	0.239/ 0.780	0.150/ 0.546	0.503**	0.693**	0.725**	0.228**
ASP26	6	8	8	2.7	2.7	2.7	0.717/ 0.742	0.660/ 0.744	0.636/ 0.723	0.033*	0.114^{ns}	0.120^{ns}	0.028^{ns}
ASP27	8	7	10	2.9	2.9	3.1	0.296/ 0.771	0.250/ 0.798	0.111/ 0.829	0.617**	0.687**	0.866**	0.030*
ASP40	12	12	6	3.0	3.0	2.3	0.400/ 0.790	0.300/ 0.803	0.000/ 0.604	0.494**	0.626**	1.000**	0.037^{ns}

Table S3. Hierarchical AMOVA (weighted average over loci) and F-statistics for *P. aspera* samples, with variance partitioned across samples and archipelagos. **, P ≤ 0.001; *ns*: non significant.

Hierarchical level	Source of variation	Sum of squares	% Variation	Fixation index
Global variance (among 3 samples)	Among samples	48.444	4.66	$F_{ST} = 0.047^{**}$
	Within samples	1287.650	95.34	
Between archipelagos (Azores vs G. Canaria)	Among archipelagos	34.695	7.39	$F_{CT} = 0.074^{**}$
	Among samples within Azores	13.749	1.58	$F_{SC} = 0.017^{ns}$
	Within populations	1287.650	91.03	

CHAPTER 4

Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic)

ABSTRACT

The uptake of natural living resources for human consumption has triggered serious changes in the balance of ecosystems. In the archipelagos of Macaronesia (NE Atlantic), limpets have been extensively exploited probably since islands were first colonized. This has led to profound consequences in the dynamics of rocky shore communities. The *Patella candei* complex includes various subspecies of limpets that are ascribed to a particular archipelago and has been the focus of several taxonomic surveys without much agreement. Under a conservational perspective, we apply morphometric and genetic analyses to test subspecies boundaries in *P. candei* and to evaluate its current population connectivity throughout Macaronesia (Azores, Madeira, and Canaries). A highly significant genetic break between archipelagos following isolation by distance was detected (F_{ST} = 0.369, P < 0.001). Contrastingly, significant genetic differentiation among islands (i.e. Azores) was absent possibly indicating ongoing gene flow via larval exchange between populations. Significant shell-shape differences among archipelagos were also detected using both distance-based and geometric morphometric analyses. Adaptive processes associated with niche differentiation and strong barriers to gene flow among archipelagos may be the mechanisms underlying *P. candei* diversification in Macaronesia. Under the very probable assumption that populations of *P. candei* from each archipelago are geographically and/or ecologically isolated populations, the various subspecies within the *P. candei* complex may be best thought of as true species using the denomination: *P. candei* in Selvagens, *Patella gomesii* in Azores, *Patella ordinaria* in Madeira, and *Patella crenata* for Canaries. This would be in agreement with stock delimitation and units of conservation of *P. candei sensu latu* along Macaronesia.

KEYWORDS: allopatric speciation, conservation genetics, gene flow barriers, limpets, phenotypic plasticity

Published as:

Faria J, Martins GM, Pita A, Ribeiro PA, Hawkins SJ, Presa P, Neto AI (2017) Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic). *Ecology and Evolution* 7(16): 6125–6140. DOI: 10.1002/ece3.3121

1. Introduction

Conservation efforts applied to human-exploited and threatened species requires a comprehensive knowledge about population structure and factors that shape differentiation within a species (Lande 1988). For instance, natural and/or human-induced barriers across a species distributional range can hasten isolation between populations by restricting the amount of individuals that can freely migrate (Barber *et al.* 2000; Kelly and Palumbi 2010). Such constraints to connectivity can lead to genetically deprived populations that, in the face of intense human-exploitation, are likely to succumb and disappear. To overcome isolation, many species have evolved life-history traits that are able to maximize dispersion and connectivity across large geographical areas (e.g. White *et al.* 2011). For instance, many widely distributed marine organisms with limited adult movement avoid population differentiation by exhibiting large population sizes with high levels of fecundity and by releasing larvae that can potentially disperse in the water column for a considerable amount of time until they reach their final destination (e.g. Faria *et al.* 2013). However, the extent of successful dispersion, which is a major determinant of population dynamics and structure, is a function of multiple and often interacting factors. For instance, an extensively and well-mixed larval pool does not necessarily lead to widespread connectivity and lack of population genetic structure over large spatial scales (e.g. Keever *et al.* 2009). The complex interplay between physical processes (e.g. coastal topography, stratified water columns, tidal forces, wind, buoyancy, surface waves, and turbulence) and life-history traits (e.g. time of spawning, larval behaviour, growth and survival rates, pelagic larval duration), often interacting at fine to mesoscales, can result in a broad range of dispersal and metapopulation connectivity patterns (see review in Cowen and Sponaugle 2009). Similarly, historical events such as past glaciations and changes in sea level can determine the contemporary distribution of populations (e.g. Portnoy *et al.* 2014).

In the last few decades, genetic methods have become a tool of excellence in investigating population differentiation estimates of a given species across its distributional range. Particularly, high-resolution nuclear markers such as microsatellites have been widely applied to conservation genetics (Guichoux *et al.* 2011) and to the identification of populations requiring prioritizing protective measures (e.g. Sandoval-Castillo and Beheregaray 2015).

The Macaronesia comprises five NE Atlantic archipelagos: Azores, Madeira, Selvagens, Canaries and Cape Verde. The region is defined as a biogeographical entity based on the existence of many shared elements in the flora and fauna among archipelagos. All are of volcanic origin and have distinct but fairly recent geological times of origin (Ávila *et al.* 2016). Patellid limpets inhabiting these archipelagos are considered a valuable resource and have been intensively exploited presumably since islands were first colonized (Santos *et al.* 1995; Côrte-Real *et al.* 1996; Hawkins *et al.* 2000). In most islands, heavy exploitation has led to dramatic decreases in limpet abundances with current populations showing clear signs of over-exploitation (Martins *et al.* 2008; Martins *et al.* 2017). Because limpet's grazing activity acts as a key process in shaping the structure and functioning of rocky shore communities (Hawkins and Hartnoll 1983), the chronic removal of limpets has led to an upward extension of turf-forming algae (see Boaventura *et al.* 2002; Martins *et al.* 2010). In fact, under

reduced numbers of limpets, algal spores can opportunistically grow to a size that allows them to escape grazing and thus form mature algal patches that are able to persist through time altering the community dynamics and energy flow (Coleman *et al.* 2006; Martins *et al.* 2010).

The intertidal limpet *Patella candei* (d'Orbigny 1840), which is exclusive to Macaronesia, occurs on rocky shores from the mid intertidal down to 5 m depth across all archipelagos except in Cape Verde where it is absent (Christiaens 1973). They are broadcast spawners with fertilization occurring in the water column. According to Martins *et al.* (1987), *P. candei* is a gonochoric species that spawns throughout the year, without synchronized resting periods. While very few studies have sentenced the pelagic larvae duration (PLD) in species of the genus *Patella* (e.g. Dodd 1957; Ribeiro 2008) with gross estimates ranging from 2 - 32 days depending on the species, temperature and settlement cues, there is still no available information about the PLD of *P. candei*. Individuals of *P. candei* have a sub-oval to stellate shell shape and an orange to greyish foot with a thin darker border. Morphological plasticity associated with specific micro-habitat conditions (i.e. substrate complexity) and environmental variation (i.e. wave exposure) is known to occur in this species (Hawkins *et al.* 1990). For instance, two distinct habitat-related morphs of *P. candei* are referred for the Azores: the 'fly limpet' and the 'smooth limpet' (Hawkins *et al.* 1990). Moreover, the morphological variation associated to each archipelago has led Christiaens (1973) to describe four distinct subspecies within *P. candei* complex: *P. candei gomesii* in Azores, *P. candei ordinaria* in Madeira, *P. candei candei* in Selvagens and *P. candei crenata* in the Canaries. This classification is not entirely supported by subsequent studies. For instance, Côrte-Real *et al.* (1996) found no differences in radular morphology and soft-body parts among archipelagos but showed that shell shape and allozyme characters from *P. candei* in Azores were clearly distinguished from *P. candei* in the Madeira and the Canaries. Weber and Hawkins (2002) also showed that *P. candei* shell shape could be distinguished among archipelagos and that allozyme retrieved two well-differentiated groups: *P. candei* from Azores and Selvagens, and *P. candei* from Madeira and Canaries. More recent research, using mtDNA, showed that samples of *P. candei* from the Azores, Madeira and Desertas (located about 25 km southeast of Madeira Island) form a well-supported group, while individuals sampled in Selvagens and Canaries always grouped together in a different clade (Sá-Pinto *et al.* 2005; 2008). The taxonomic status of Macaronesian limpets thus remains unclear with much controversy as to whether *P. candei* from the different archipelagos should be given a specific status.

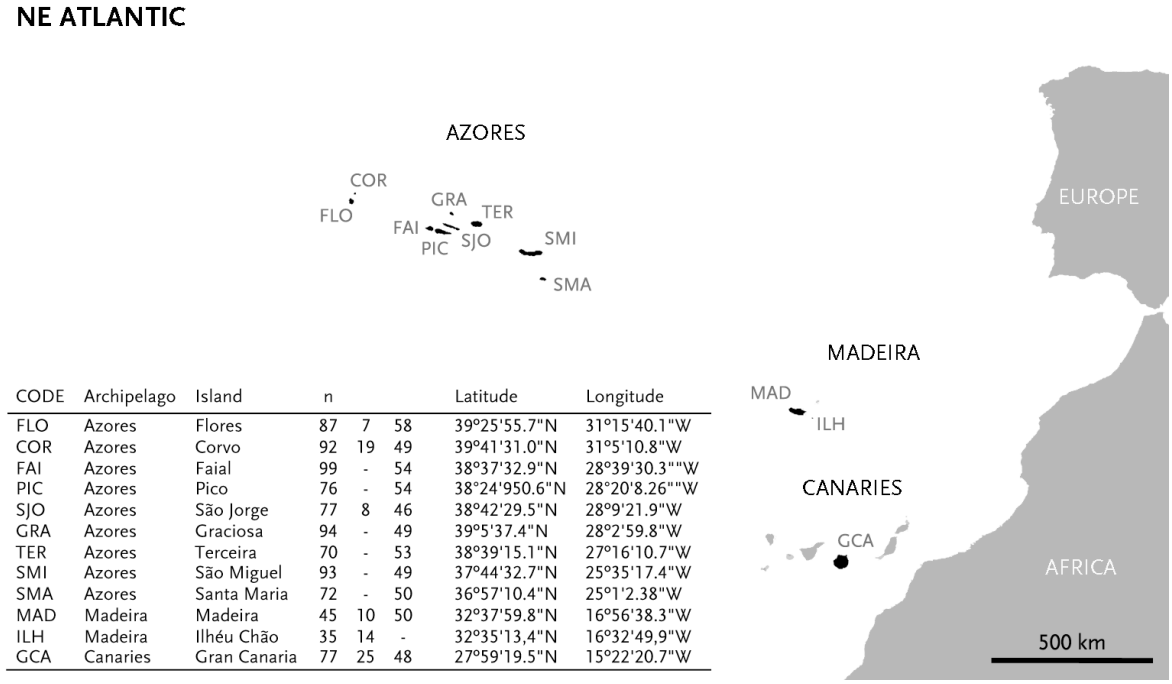
In this study, we use morphometric and molecular genetic methods to assess the existence of distinct groups of *P. candei* across Macaronesia, testing the subspecies boundaries within the species. At multiple spatial scales, we evaluate the degree of contemporary connectivity among populations and hypothesize that populations geographically closer to each other are likely more related and connected via larval dispersion. Besides the assessment of genetic diversity and structure of *P. candei* populations across archipelagos, and given the importance of defining conservation units in fisheries planning (Hawkins *et al.* 2016), we provide discussion and guidance about protective measures of such threatened marine resource, highlighting the importance of considering levels of genetic diversity in populations as well as their uniqueness.

2. Methods

2.1. Morphometric analyses

2.1.1. Distance-based analyses

Individuals of *P. candei* were collected across the Macaronesia archipelagos of Azores, Madeira, and Canarias, in 12 different islands (n = 917) during the summer of 2011 (Fig. 1). In all islands, individuals were collected on intertidal platforms. The soft tissue of all individuals was carefully removed for genetic analyses, and the shells were marked individually. Shell morphometry was examined using the procedure described in detail by Cabral (2007). In summary, five distances were measured on each shell: shell length (SL), shell maximum width (SW), shell width at apex (SWA), distance from apex to anterior tip (SAA), and shell height (SH). These distances were then used to calculate base ellipticity (BE), base eccentricity (BEC), conicity (CO), and cone eccentricity (CE) for each individual shell (see Cabral 2007 for further details). Shells were measured using a Vernier caliper with a precision of 0.1 mm. Individuals with signs of shell damage or with clear home fitting deformation were discarded from the analyses. The four variables were inspected for collinearity and removed from the analyses when collinearity was high (r > 0.3).



CODE	Archipelago	Island	n			Latitude	Longitude
FLO	Azores	Flores	87	7	58	39°25'55.7"N	31°15'40.1"W
COR	Azores	Corvo	92	19	49	39°41'31.0"N	31°5'10.8"W
FAI	Azores	Faial	99	-	54	38°37'32.9"N	28°39'30.3""W
PIC	Azores	Pico	76	-	54	38°24'950.6"N	28°20'8.26""W
SJO	Azores	São Jorge	77	8	46	38°42'29.5"N	28°9'21.9"W
GRA	Azores	Graciosa	94	-	49	39°5'37.4"N	28°2'59.8"W
TER	Azores	Terceira	70	-	53	38°39'15.1"N	27°16'10.7"W
SMI	Azores	São Miguel	93	-	49	37°44'32.7"N	25°35'17.4"W
SMA	Azores	Santa Maria	72	-	50	36°57'10.4"N	25°1'2.38"W
MAD	Madeira	Madeira	45	10	50	32°37'59.8"N	16°56'38.3"W
ILH	Madeira	Ilhéu Chão	35	14	-	32°35'13,4"N	16°32'49,9"W
GCA	Canaries	Gran Canaria	77	25	48	27°59'19.5"N	15°22'20.7"W

Figure 1. Map of sampling locations for *Patella candei* collected from the Macaronesian archipelagos of Azores, Madeira, and Canaries (NE Atlantic). *n* columns indicate the number of individuals used for distance-based morphometrics, geometric morphometrics, and genetic analyses, respectively.

Examination of the spatial variation in shell morphometry among archipelagos was analysed using a two-way fully hierarchical analysis of variance (ANOVA) with the following factors: archipelago (fixed) and island (random and nested within archipelago). Analyses were performed using PERMANOVA+ (Anderson *et al.* 2008) based on Euclidean distances and using 999 permutations. PERMANOVA is a

permutational-based test and produces results analogous to ANOVA when based on Euclidean distances (Anderson 2001). This test was preferred over classical ANOVA because it allows the use on unbalanced designs (e.g. different number of islands per archipelago). When needed, SL was used as a covariate in order to adjust for differences in morphometry with animal size. Prior to analysis, PERMDISP was used to test for homogeneity of multivariate dispersions. All analyses were performed on untransformed data. Principal component analysis (PCA) was performed to determine the linear combination of morphometric descriptors that account for the variation in the data. Shell-shape variation between *P. candei* morphotypes ("fly" and "smooth") from Azores was also accessed by means of ANOVA. Given the difficulties in sorting all individuals into the "fly" and "smooth" categories, only distinctive shells of each morphotype were used in the analysis (n = 346). Note, however, that this criteria may upward bias the degree of differentiation between morphotypes.

2.1.2. Geometric morphometric analyses

A total of 83 *P. candei* collected across archipelagos were used for landmark-based geometric morphometric analysis (Bookstein 1991; Fig. 1). For imaging, shells were positioned in a light background and digital high-resolution images of the dorsal surface were captured using a CANON EOS 600D camera mounted on a tripod to maintain the distance for all samples and to ensure that the lens was parallel to the surface examined. The anterior–posterior axis of each specimen was identified using the scars from each individual body left on the ventral surface of the shell. A fan was used to position each specimen along such axis and ensuring that the apex of each shell coincided with the vertical line of the fan (Fig. S1). A tps file of each archipelago specimens was created using tpsUtil, and tpsDig2 (Rohlf 2015) was used to place a total of 37 landmarks on the shell apex and on the intersection of the fan and shell of each sample specimen image. Except for the shell apex landmark, all other points (at the shell border) do not necessarily represent homologous landmarks from a development point of view, but can be used to decompose objectively the shell shape of limpets. These points are referred to as semi-landmarks and can be used to capture information about curvature (Gunz and Mitteroecker 2013). Specimens were then aligned using a Generalized Procrustes Analysis (GPA) (Rohlf and Slice 1990) to remove all the differences due to translation, rotation, and scale (Bookstein 1991). In this process, semi-landmarks are allowed to slide along their tangent directions so as to minimize the bending energy between each specimen and the reference form. The resulting aligned Procrustes coordinates represent the shape of each specimen. For more details on geometric morphometric methodologies using landmarks, see Bookstein (1991), Zelditch *et al.* (2012), Adams *et al.* (2013). Centroid size (CS), given by the square root of the sum of squared distances of a set of landmarks from their centroid, was also calculated (Rohlf and Slice 1990). A Procrustes permutation analysis of variance (Procrustes ANOVA) performed with a residual randomization permutation procedure (Collyer *et al.* 2015; Adams *et al.* 2016) was used to determine patterns of shell shape variation between archipelagos. The aim is to test groups (archipelagos) considering the influence of phenotypic change associated with body size (i.e. body size was calculated from landmark configurations as centroid size). Actually, the Procrustes ANOVA between

shape and size, using CS is a way to assess interspecific allometry (Villegas *et al.* 2002). Because allometry was detected among archipelagos (see section 3), the full dataset was divided in two: one with small limpets (SMALL) and one with big limpets (BIG). Principal components analysis (PCA) was used to provide a graphical depiction of patterns of shape variation across the two datasets. Thin plate splines were used to provide a visual representation of the shape changes between each group mean and overall consensus configuration. All analyses and graphical representations were performed in R (R Core Team 2014) using the packages GEOMORPH (Adams and Otarola-Castillo 2013). Geometric morphometric analysis was also used to determine shell shape variation between the "fly" and "smooth" *P. candei* morphotypes from Azores (n = 23 and n = 34, respectively).

2.2. Genetic analysis

2.2.1. Sampling and laboratory protocols

A total of 560 individuals of *Patella candei* from the three archipelagos were used for genetic analysis (Fig. 1). Upon collection, limpets were preserved in 96% ethanol and frozen for later processing. At the laboratory, samples were subject to DNA extraction from the foot muscle tissue using the E.Z.N.A. Mollusc DNA extraction kit and following the manufacturer' instructions. The quality and quantity of DNA extractions were assessed using a Nanodrop spectrophotometer (Thermo Scientific). All individuals were genotyped at 12 microsatellite loci using the primer pairs and following the amplification protocol described in Faria *et al.* (2016). Briefly, microsatellites were amplified in three distinct multiplex PCRs (PcaMix1: loci CAN18, CAN25, CAN27, CAN53; PcaMix2: loci CAN9, CAN26, CAN32, CAN40; PcaMix3: loci CAN23, CAN33, CAN56, CAN60) on 10 µl reactions containing ~30 ng DNA template, 1 × Qiagen™ Multiplex PCR Kit, 0.5 – 1.2 µmol/L of each primer and ddH_2O. Genotyping was performed on an ABI 3730 (Applied Biosystems) automated DNA sequencer using an internal size standard (GeneScan™ 500Liz®, Applied Biosystems) for accurate sizing and GENEMAPPER™ v.4.2 (Applied Biosystems) was used for allele calling.

2.2.2. Genetic variation

Genetic diversity estimates such as allele frequencies and observed and expected heterozygosities (H_O and H_E) were estimated in GenAlEx v.6.5 (Peakall and Smouse 20012). The fixation index (F_{IS}), linkage disequilibrium, and deviations from the Hardy–Weinberg equilibrium (HWE) were tested in GENEPOP v.4.2 (Raymond and Rousset 1995). Allelic richness [$A_R(g)$] and private allele richness [$A_P(g)$] were estimated using the rarefaction method implemented in ADZE v.1.0 (Szpiech *et al.* 2008). Whenever needed, the false discovery rate (FDR) control was employed to account for multiple testing (Verhoeven *et al.* 2005). The presence and frequency of null alleles was tested for each locus using MICROCHECKER v.2.2.3 (van Oosterhout *et al.* 2004) and FREENA (Chapuis and Estoup 2007), respectively.

2.2.3. Genetic differentiation and population structure

Pairwise F_{ST} estimates among populations were calculated using FSTAT v.2.9.3 (Goudet 1995), and departures of F_{ST} from the null hypothesis of panmixia were evaluated via a permutation test (1 000 iterations). The effect of null alleles in F_{ST} estimates was assessed by comparing F_{ST} before and after correction for null alleles using the excluding null alleles (ENA) method implemented in FREENA. Genetic differentiation between populations was also determined using the D_{est} estimator (Jost 2008) implemented in the R package DEMETICS v.0.8.4 (Gerlach *et al.* 2010), and P-values were estimated by bootstrap analysis (1 000 replicates). For all analyses involving multiple tests, significance levels were adjusted by the FDR method.

The model-based approach implemented in STRUCTURE v.2.3.3 (Pritchard *et al.* 2000) was used to identify the most likely number of populations (K) and assign individuals to genetic clusters. Assignment is conducted in ways that minimize deviations from Hardy–Weinberg and linkage equilibrium within each cluster. No particular population structure was assumed a priori (LOCPRIOR = 0), and ten independent runs were carried out for each value of K (1–11). Length of the burn-in period was set to 1×10^5 followed by 5×10^5 Markov chain Monte Carlo (MCMC) iterations. Correlated allele frequencies and admixed populations were assumed. Modifications in such parameters produced consistency and did not change the final results. Selection of the most likely number of genetic clusters (K) was based on checking the posterior probability of the data for a given K (Pritchard *et al.* 2000) and also by looking at the second-order rate of change in probability between successive *K* values as described in Evanno *et al.* (2005) and implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). In systems with hierarchical population structure, STRUCTURE typically best resolves the highest level of population subdivision (Evanno *et al.* 2005). Thus, in order to resolve lower levels of subdivision, structure analyses were also conducted separately for each cluster identified. Therefore, two additional STRUCTURE analyses using the same settings were used to identify potential within-cluster structure. The best K was determined as previously described.

A discriminant analysis of principal components (DAPC) was also performed to identify and describe clusters of genetically related individuals (Jombart *et al.* 2010). DAPC has been shown to perform generally better than STRUCTURE at characterizing population subdivision (Jombart *et al.* 2010). DAPC is a multivariate analysis that integrates principal component analysis (PCA) together with discriminant analysis to summarize genetic differentiation between groups. DAPC is free of assumptions about Hardy–Weinberg equilibrium or linkage disequilibrium and provides graphical representation of divergence among populations. DAPC was performed with and without using prior group information using the R package ADEGENET (Jombart 2008). All STRUCTURE and DAPC analyses were conducted upon removal of non-amplifying loci.

Tests for genetic differentiation among archipelagos were also conducted using analysis of molecular variation (AMOVA) in ARLEQUIN v.3.5.1.3 (Excoffier and Lischer 2010). Genetic variation among archipelagos (F_{CT}), among populations within archipelagos (F_{SC}) and within populations (F_{ST}) was assessed, and significance of F-statistics was tested using 10 000 permutations. Estimates of genetic

differentiation were also determined among the “fly” and “smooth” morphotypes of *P. candei* from Azores.

2.2.4. Isolation by distance and gene flow

To test for isolation by distance (Wright 1943), linearized F_{ST} transformation (F_{ST} / [1 − F_{ST}]) was regressed onto the natural log of geographic distances (GD; Rousset 1997). Regression with GD was also performed with the differentiation estimator D_{est} matrix. Regression analyses were performed in R and tested for significance with a Mantel permutation procedure. Moreover, given the heterogeneous nature of samples, the Monmonier’s maximum difference algorithm implemented in BARRIER v.2.2 was used to highlight geographical features associated with genetic discontinuities among populations (see Manni *et al.* 2004 for method details). Analyses were conducted using pairwise F_{ST} values, and statistical confidence for each identified barrier was evaluated using 100 bootstrap replicates that were simulated using the package diveRsity in R. Analyses were also conducted separately for each amplifying microsatellite locus.

Recent migration rates (m) among populations/clusters identified in STRUCTURE were estimated using the Bayesian multilocus genotyping procedure implemented in BAYESASS v.3.0 (Wilson and Rannala 2003). Analyses were only conducted among archipelagos due to the lower accuracy of BAYESASS when migration rates are high and genetic differentiation is low (see section 3) (Faubet *et al.* 2007). The program was run for 3×10^6 MCMC iterations with sampling at every 1 000 iterations, of which 10^6 iterations were discarded as burn-in. Delta values for allele frequency, migration rate, and inbreeding were adjusted so that the accepted numbers of changes were 40% – 60% of the total number of iterations. Ten MCMC runs with different initial seeds were carried out in order to maximize convergence and mixing. The Bayesian deviance was used as an optimality criterion to find the run with the best fit (Faubet *et al.* 2007). Deviance was calculated from the trace file using the R-script provided by Meirmans (2014).

Contemporary gene flow was also estimated using the F-model on BIMr program (Faubet and Gaggiotti 2008). This software can estimate migration rates and detect migrants (within the last generation) at a lower level of population differentiation compared to BAYESASS (Faubet and Gaggiotti 2008). In addition, BIMr can identify the environmental factors that are more likely to explain the observed patterns using a generalized linear model. The method employs a Bayesian approach and Markov chain Monte Carlo (MCMC) techniques to make inferences of recent gene flows in subdivided populations (Faubet and Gaggiotti 2008). Preliminary trials included all populations but because population-specific F_{ST} values below 0.01 can be problematic for parameter estimation, analyses were performed on samples grouped according to the Bayesian clustering analyses results. Also, analyses were conducted with and without removing loci that failed to amplify in some populations and/or exhibited null alleles. Often considered one of the main factors in determining gene flow in many species, the geographic distance between samples was included as the environmental variable. A total of 20 independent replicate runs were performed. Each MCMC was run for a total of 3.53×10^6 iterations, which included 30 short pilot runs of 1 000 iterations each in an effort to obtain

acceptance rates between 25% and 45%. The next 15 × 10^5 iterations were discarded as burn-in, and a total of 20 000 samples were collected from each of the 20 replicates using a thinning interval of 100 iterations, using default settings. The posterior probabilities were evaluated for the run with the lowest Bayesian deviance (given by the assignment component of the total deviance: D_{assign}) (Faubet *et al.* 2007; Faubet and Gaggiotti 2008). The mean, mode (point estimate), and 95% highest posterior density intervals (HPDI) for migration rates were recorded.

3. Results

3.1. Distance-based morphometrics

Shell samples of *P. candei* ranged in size (SL) between 1.35 and 6.35 cm, with a mean size of 3.25 ± 0.03 cm (mean ± SE). Although variable across islands (ANOVA, P < 0.001), mean SL did not differ among archipelagos (ANOVA, P > 0.05, Fig. S2, Table S1) but was significantly correlated with the remaining distance measures (SW, SWA, SAA, and SH) and also with BE (Table S2). Hence, only SL was used for analyses and considered as a covariate in the analysis of spatial variation of BE. Among the morphometric descriptors, conicity (CO) was positively correlated with BEC and CE and was therefore selected for analysis together with BE. Significant variation in the shape, as given by analyses of the descriptors CO and BE, was found at the scale of islands and among archipelagos (ANOVA, P < 0.05, Tables S3 and S4). Variation in shell BE among archipelagos, although affected by size (SL), was relatively higher than variation among islands (Fig. 2). Similarly, for conicity, the largest proportion of the variability was found at the scale of archipelagos (Fig. 2). The first principal component (PC1) described 62.2% of the total variation, with the remaining variation (%) being accounted by the second principal component (PC2). The most important variable integrated by the first and second components was conicity and BE, respectively. The PCA showed that shell conicity can better distinguished shells from different archipelagos, whereas shell BE was mostly associated with differences within archipelagos (Fig. 3). Significant shell shape variation was also detected between the two *P. candei* morphotypes in Azores (ANOVA, P < 0.01). Differences were only detected for shell conicity, with “fly limpets” being more conical then “smooth limpets.”

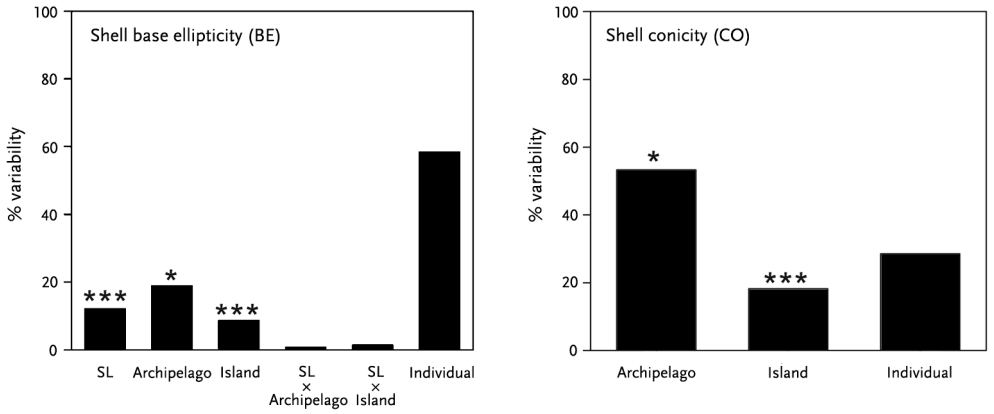


Figure 2. Components of variability for shell base ellipticity (BE) and shell conicity (CO) in *Patella candei* across archipelagos; SL stands for shell length. Significance: *P < .05, *** P < .001 (see Tables S3 and S4 for ANOVA terms).

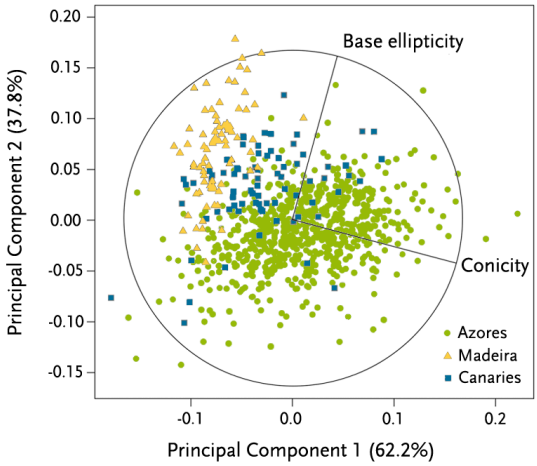


Figure 3. Principal component plot for shell morphometric descriptors, conicity (CO) and base ellipticity (BE), in *Patella candei* from the Macaronesian archipelagos of Azores (N = 760), Madeira (N = 80), and Canaries (N = 77); eigenvector PC1: BE (0.258), CO (0.966); PC2: BE (0.966), CO (−0.258).

3.2. Geometric morphometrics

The Procrustes ANOVA analysis on the full shell shape dataset revealed a significant interaction between archipelago and size, indicating an allometric growth in *P. candei* (Table 1). Moreover, the null hypothesis for common allometries (parallel slopes) among archipelagos was rejected ($F = 5.364$, $P < 0.001$). The amount of shape change per unit of size change differed among archipelagos and was greater in Azores (indicated by the lengths of slope vectors) (Table 1; Fig. S3). Yet, shape trajectories and the way shapes change were only significantly different between Canaries and Madeira (indicated by the angles between slope vectors) (Table 1; Fig. S3). This corresponds to contrasting local deformations in particular parts of the landmark configuration associated with size change in these two archipelagos (e.g. note the changes in the shell apex landmark in Madeira and Canaries; Fig. S4). Significant differences in shell shape unrelated to size were detected among archipelagos for the two subsets (SMALL and BIG; Table S5). For both datasets, pairwise comparisons showed that Azorean and Canaries shells could not be distinguished (Table S5). The first two principal components of the Procrustes shape variables for each dataset accounted for 52 and 53% of the total sample variation, respectively (Fig. 4). A generalized overlapping in the scatter of data was found, mostly between Azores and Canaries samples. Intraspecific variance was greatest in smaller shells from Madeira and Canaries, with individuals from these archipelagos occupying a much wider range of shape space than samples from Azores. Deformation grids for both SMALL and BIG datasets indicate that shell goes from a clear round shape to a more ridged and pointy look-alike shape along CV1 (left to right) (Fig. 4). On the same direction, the shell apex gets closer to the anterior margin of the shell. Similarly, the anterior end of the shell gets narrower along such axis. These shape changes are mostly associated and illustrate shell shape differences between Azores/Canaries and the Madeira samples. Whereas Azorean shells are oval with a smoother margin, the Canaries samples exhibit some ridges along their shell border. The pentagon look-alike shape of *P. candei* in Madeira stands out from the remaining archipelagos (Fig. S5). As for shell shape variation between *P. candei* morphotypes (“fly” and “smooth”) from Azores, the Procrustes ANOVA analysis revealed a significant interaction between morphotype and size, which is indicative of allometric growth. The null hypothesis for common allometries (parallel slopes) among morphotypes was

rejected (F = 7.459, P < 0.01), and differences were detected in the amount of shape change per unit of covariate change (size) between morphotypes; shape change per size unit is higher in “fly” limpets (Table S6). Overall shape of *P. candei* in Azores is oval for both morphotypes but the shell apex in “fly” limpets tends to get closer to the anterior margin of the shell (Fig. S6).

Table 1. Procrustes ANOVA examining differences in patterns of shell shape variation among archipelagos (10 000 random permutations). Centroid size (CS) was used as a covariate. Slope pairwise comparisons among archipelagos are shown; contrasts in slope vector length and angles between slope vectors are shown in upper and lower diagonal, respectively. Effect sizes (Z) are standard deviations of the observed size.

	df	R^2	Z	F
SIZE *log(CS)*	1	0.136	9.814	15.480***
ARCHIPELAGO	2	0.149	6.695	8.502***
SIZE *log(CS)* × ARCHIPELAGO	2	0.039	2.146	2.226***
Total	82			
Slope pairwise comparison	AZORES	CANARIES	MADEIRA	
AZORES	-	0.121*	0.118*	
CANARIES	47.4	-	0.097*	
MADEIRA	45.8	50.3**	-	

* P < 0.05, *** P < 0.001; effect sizes (Z) are standard deviations of the observed size.

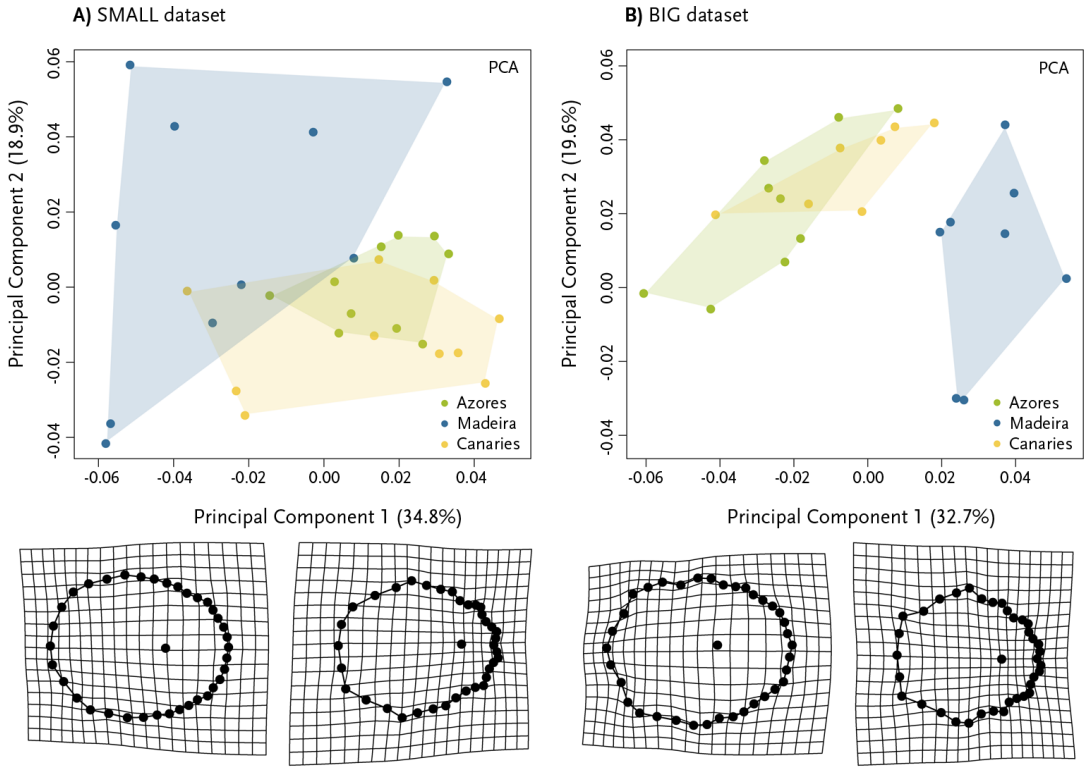


Figure 4. Principal component analyses of Procrustes coordinates derived from the first two principal components (PC1 and PC2) for the (A) SMALL dataset and (B) BIG dataset. Convex hulls are drawn to show the area of the morphospace occupied by each archipelago; the thin-plate spline deformation grids display the shape of specimens at the ends of the range of variability along each PC1.

3.3. Genetic analysis

A total of 138 alleles were observed across the 12 loci examined, ranging from six in CAN26 and CAN56 to 22 alleles in CAN18 (Table S7). Five loci failed to amplify in individuals from Madeira and Canaries: Loci CAN9, CAN26, and CAN33 did not amplify at all in these two populations and two additional loci failed to amplify in more than 30% of both samples (Table S8). Since microsatellite markers were developed using *P. candei* from the Azores (see Faria *et al.* 2016), such amplification failure suggests a high genetic differentiation between Azores and the remaining archipelagos. Multilocus mean allelic richness with rarefaction was similar across populations and ranged from 3.6 (GCA) and 4.7 (FAI). Mean number of private alleles was greater in the MAD population (A_P[30] = 1.14). Observed heterozygosity frequencies (H_O) were relatively low and ranged from 0.157 to 0.364, while expected (H_E) heterozygosity frequencies ranged from 0.304 to 0.484 (Table S7). No significant linkage disequilibrium was detected for any pairs of loci. Except for loci CAN23 and CAN53, all other loci deviated from Hardy–Weinberg equilibrium (HWE). Significant locus-specific inbreeding coefficients (F_{IS}) ranged from 0.073 to 0.746 denoting a heterozygous deficit in such loci. Overall, significant F_{IS} values were often ascribed with the presence of null alleles. To check for any bias in the results, loci with a presence of null alleles > 10% were removed from the analysis. Such removal did not affect genetic differentiation results (data not shown) and unless stated otherwise, all loci were included in subsequent genetic analyses. In fact, the influence of null alleles has been shown to be marginal when compared to other factors such as number of loci and strength of population differentiation (Carlsson 2008). Pairwise comparisons of F_{ST} and D_{est} indicated high and significant genetic differences among archipelagos but not within archipelago (i.e., between islands in Azores) (Table 2). Both indices were highly correlated (Pearson's correlation 0.99, $P < 0.001$) (Fig. S7). Furthermore, F_{ST} values before and after correction for null alleles using the ENA method did not differ considerably (Table S9).

Table 2. Pairwise estimates of $F_{ST\text{-}FREENA}$ (below diagonal) and Jost's D_{est} (above diagonal) between all populations sampled for *Patella candei* (see Fig. 1 for population codes)

	FLO	COR	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA
FLO	-	0.020	0.010	0.000	0.018	0.005	0.002	0.022	0.033	**0.505**	**0.594**
COR	0.008	-	0.006	0.016	0.009	0.008	0.005	0.006	0.010	**0.492**	**0.588**
FAI	0.004	0.002	-	0.007	0.007	0.005	0.014	0.010	0.015	**0.481**	**0.577**
PIC	0.000	0.008	0.000	-	0.004	0.000	0.003	0.004	0.008	**0.504**	**0.596**
SJO	0.004	0.004	0.000	0.001	-	0.000	0.013	0.001	0.006	**0.474**	**0.587**
GRA	0.000	0.004	0.000	0.000	0.000	-	0.008	0.004	0.002	**0.494**	**0.588**
TER	0.005	0.010	0.011	0.009	0.009	0.006	-	0.014	0.020	**0.500**	**0.597**
SMI	0.005	0.007	0.000	0.000	0.000	0.000	0.009	-	0.000	**0.493**	**0.594**
SMA	0.006	0.008	0.006	0.001	0.002	0.000	0.010	0.000	-	**0.499**	**0.597**
MAD	**0.319**	**0.307**	**0.296**	**0.308**	**0.286**	**0.308**	**0.336**	**0.314**	**0.323**	-	**0.203**
GCA	**0.388**	**0.376**	**0.360**	**0.373**	**0.361**	**0.377**	**0.405**	**0.385**	**0.392**	**0.127**	-

Significant values after FDR correction are shown in bold.

The STRUCTURE and DAPC analyses provided support for the genetic differentiation indicated by F_{ST} and D_{est}. In fact, the genetic structure inferred from the 560 individuals of *P. candei* and the 12 microsatellite loci using the Bayesian model-based clustering algorithm, and the model-free DAPC clustering algorithm, provided similar results (Fig. 5). Two clusters (K = 2) were identified when considering all locations, with Azores samples being separated from the Madeira and Canaries populations. Similarly, two well-defined clusters were retrieved on the Madeira and Canaries assignment analysis. Both STRUCTURE and DAPC also suggested population homogeneity for *P. candei* in Azores (K = 1). In this case, for the STRUCTURE analysis, the estimated membership of individuals to any given cluster was roughly symmetric (~1/K in each population), indicating that individuals in Azores are widely admixed and belong to a single panmictic population (Fig. 5). Results from the Bayesian clustering analysis and DAPC were consistent with those obtained using AMOVA, which detected significant genetic differences among archipelagos. According to the AMOVA, 36.6% of the total genetic variation was found among archipelagos ($P < 0.001$), 0.2% among populations of the same archipelago ($P = 0.268$), and 63.1% within populations ($P < 0.001$). Furthermore, a significant positive relationship was observed between genetic differentiation estimators (F_{ST} and D_{est}) and geographic distance (Fig. 6). However, such relationship (IBD) is only endorsed among archipelagos and not within archipelago (i.e. populations from Azores are genetically undistinguished) (Fig. 6). The Monmonier's algorithm in BARRIER revealed the existence of strong spatial barriers to

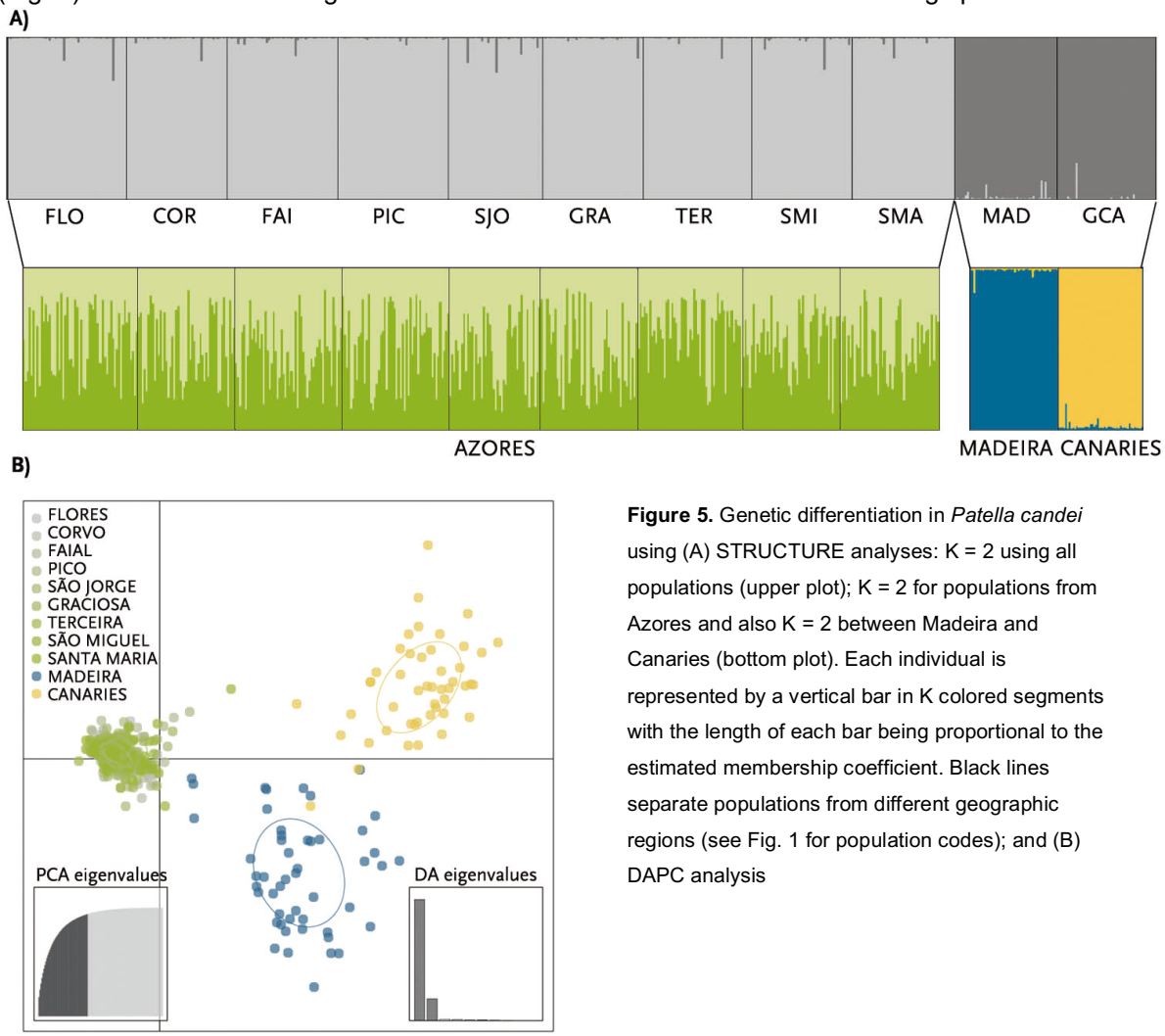


Figure 5. Genetic differentiation in *Patella candei* using (A) STRUCTURE analyses: K = 2 using all populations (upper plot); K = 2 for populations from Azores and also K = 2 between Madeira and Canaries (bottom plot). Each individual is represented by a vertical bar in K colored segments with the length of each bar being proportional to the estimated membership coefficient. Black lines separate populations from different geographic regions (see Fig. 1 for population codes); and (B) DAPC analysis

gene flow among archipelagos (Fig. 7). No genetic differentiation was found between *P. candei* morphotypes from Azores ($F_{ST\text{-}FREENA}$ = 0.001 and D_{est} = 0.002, both non-significant; STRUCTURE: best K = 1).

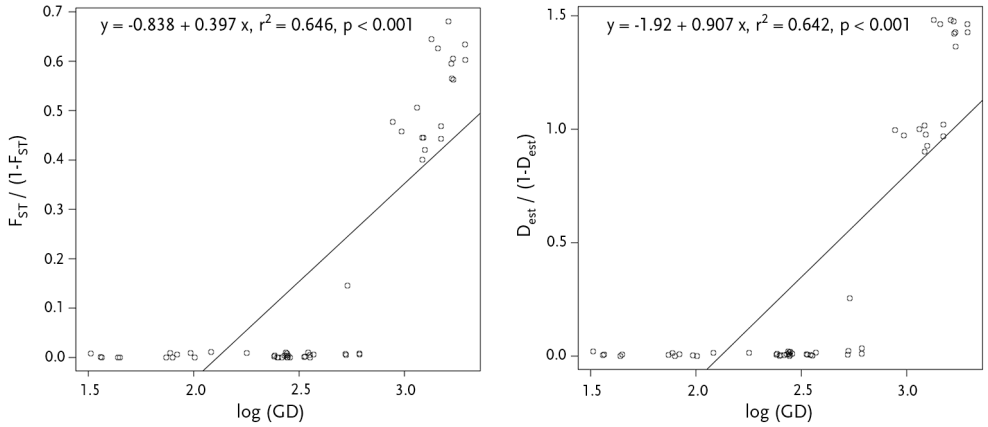


Figure 6. Regression between genetic distances F_{ST} / (1−F_{ST}) (top plot) and D_{est} (bottom plot), with natural log geographical distances. Whereas a strong signal of IBD is observed among archipelagos, basal pairwise points in both graphs indicate that genetic differentiation between geographically separated islands within archipelago is absent (i.e. Azores).

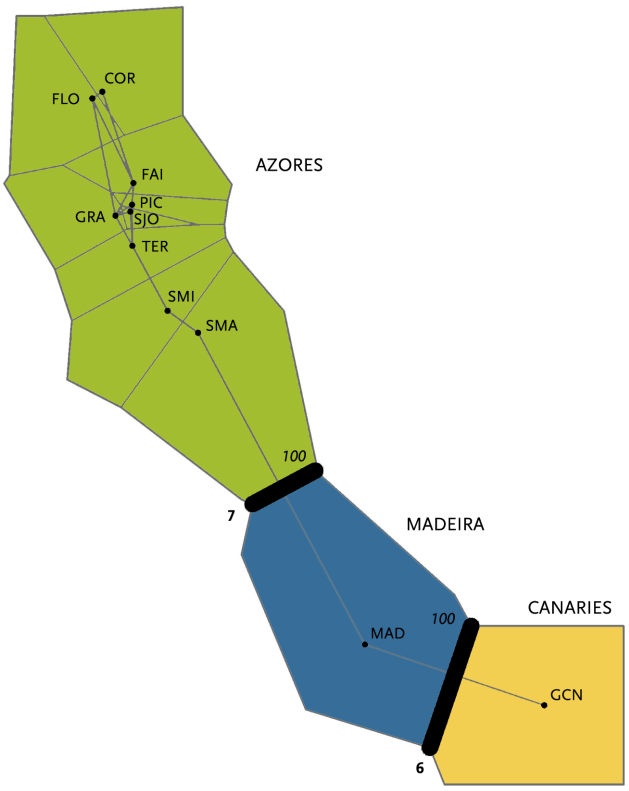


Figure 7. Areas of genetic discontinuity identified with BARRIER using the Monmonier's algorithm. Barriers to gene flow are indicated by thick black lines; the smaller adjacent numbers relate to the proportion of times the barrier was observed across 100 bootstrap replicates; bolded figures indicate the number of loci out of nine supporting the observed barriers. Only those barriers supported by more than half the loci set and high bootstrap values (> 50%) are shown.

The results of the migration rates estimated in BAYESASS suggest a consistent restriction in contemporary gene flow between archipelagos (Table 3). Yet, from the individual assignments, two individuals sampled in Madeira had posterior probability estimates (0.13 and 0.44, respectively) as second-generation migrants from Canaries. One individual sampled in the Canaries had a probability of 0.24 to be a first-generation migrant from Madeira (Table S10). Estimates of current gene flow rates from BIMr for populations of *P. candei* were consistent across all preliminary trials and runs and showed mean values very close to 0 (Table 3). Such small estimates suggest effectively the absence of contemporary gene exchange among archipelagos. Moreover, the highest posterior probability was assigned to the model excluding distance as a factor ($P_{[none]}$ = 57.3; $P_{[distance]}$ = 42.7). The observed migration rates were independent of geographic distance as migration between archipelagos was similar and virtually absent.

Table 3. Contemporary gene flow in *Patella candei* between archipelagos as depicted with the average posterior distribution of migration rates from (A) BAYESASS (and 95% CI) and (B) BIMr (95% HPDI)

From/into	AZORES	MADEIRA	CANARIES
A) BAYESASS v.3.0			
AZORES	0.999 (0.997-1.000)	0.006 (0.000-0.019)	0.007 (0.000-0.020)
MADEIRA	0.001 (0.000-0.002)	0.979 (0.953-1.000)	0.009 (0.000-0.024)
CANARIES	0.001 (0.000-0.002)	0.015 (0.000-0.037)	0.985 (0.963-1.000)
B) BIMr			
AZORES	1.000 (0.993-1.000)	0.000 (10^{-5}-0.003)	0.000 (10^{-17}-0.005)
MADEIRA	0.000 (0.000-0.051)	0.999 (0.910-0.996)	0.000 (0.000-0.082)
CANARIES	0.000 (0.000-0.0298)	0.000 (10^{-5}-0.034)	1.000 (0.964-0.998)

4. Discussion

Here, we investigate current signs of population differentiation and connectivity in the *P. candei* complex across Macaronesia (NE Atlantic). Our results revealed highly structured populations among archipelagos, which are likely associated with strong barriers to gene flow. Although isolation by distance (IBD) was detected among archipelagos, connectivity within archipelago (i.e. Azores) does not follow IBD, with genetic homogeneity among populations (i.e. islands) being maintained possibly via broad larval exchange. Moreover, shell shape differences among archipelagos were also detected and are likely the consequence of mixed effects of historical vicariance and re-colonization events, genetic drift, and local adaptations.

Limitations to this study include the lack of samples from some Macaronesia islands (i.e., no samples from Selvagens and single samples from Canaries and Madeira), so that results among archipelagos cannot be fully explored in comparison with patterns within archipelagos. Despite this shortcome, the inclusion of samples from Madeira and Canaries allowed showing that the *Patella candei* complex across Macaronesia is highly differentiated and that each subspecies (or species) endorsed to a given archipelago should be treated as a single conservation unit.

4.1. Shell shape variation between archipelagos

There have been few attempts to describe and distinguish limpet species and/or morphotypes using shell morphometry (e.g. Denny 2000; Cabral 2007). The difficulties of such methods rely on the fact that limpet shells have a sub-oval shape without any clear external homologous landmarks (except for the shell apex) or readily identifiable morphological features. Furthermore, due to the strong influence of certain environmental factors (i.e. wave exposure, substrate complexity, predation) many organisms, including limpets, exhibit high morphological plasticity (Branch and Marsh 1978; Lowell 1986; Sokolova and Berger 2000; Guerra-Varela *et al.* 2009; Harley *et al.* 2009). The advent of modern techniques such as geometric morphometrics has furthered the ability of researchers to differentiate species and or specimens based on morphological characters (e.g. Baylac *et al.* 2003; Ruane 2015; Davis *et al.* 2016). In such sense, whereas distance-based methods are considered rather more simplistic in detecting shape differences between samples, the geometric analysis allows a more detailed recognition and assessment of how such shape varies with shell growth. Despite intrinsic different, generally, both methods allowed to distinguish *P. candei* morphotypes among and within archipelagos. The differences found between both methods are likely associated to (1) the fact that distance-based data contain relatively little information about shape because many of the measurements overlap and/or are correlated to each other, and shape can only be derived from ratios among particular measurements and (2) the inclusion of shell height to the distance-based method provides a third dimension, which is absent in geometric morphometrics that only considers a two-dimensional reduction of shape in current analyses. Depicting the results, distance-based methods show that samples from Azores exhibit a more conical and elliptical shell shape compared to samples from the Canaries and Madeira, with the later descriptor being influenced by size. In fact, shell shape allometries derived from geometric morphometrics differ among archipelagos, and globally, differences are mostly found between Madeira samples and the remaining archipelagos; both small and large limpets from Azores and Canaries are more similar between them than with samples from Madeira. Whereas genetic data suggest a closer relationship between *P. candei* from Madeira and Canaries populations (see sections 3 and 4), shell shape differences are more thinned between *P. candei* from Azores and Canaries. This variation in morphology does not need to be necessarily consistent with genetic variation, especially because neutral markers such as microsatellites can be subject to distinct evolutionary forces than selected loci (McKay and Latta 2002).

Shell shape variation in *P. candei* among archipelagos seems to be associated with mixed effects of ancient vicariance events, genetic drift and particular local adaptations under restrictive gene flow that acted together to produce such dissimilarities. For instance, the genetic stochasticity associated with distinct evolutionary histories such as time of colonization in each archipelago and/or specific retraction and expansion demographic events under limited gene flow may have contributed to the observed morphological variation. These mechanisms are likely responsible for the occurrence of several patellid species with considerable differentiation in shell shape on similar habitats across Macaronesia. For example, in Madeira, *P. candei* and *Patella piperata* have an overlapping intertidal

distribution, even though the shell of *P. piperata* is more round shaped and exhibits small black granules along its shell. Historical and/or contemporary diverging local selection pressures, and subtle differences in temperatures, hydrodynamic forces, substrate composition, community assemblages, and available competitors among archipelagos are also likely to have played a key role in determining variation in limpet shell shape on such remote islands. Moreover, the adaptive phenotypic plasticity associated with multiple environmental conditions, which is common in many intertidal molluscs (e.g. Wolf *et al.* 1997; Trussell 2000), can also determine geographic variation. Within the Azorean archipelago, such phenotypic plasticity is evident in the two well-recognized habitat morphs: The "fly limpet" which is highly conical and commonly found upper on the shore, mostly on more rugose surfaces; and the "smooth limpet" which is more flattened and associated to surfaces highly exposed to hydrodynamic forces (Hawkins *et al.* 1990). Under particular circumstances, such phenotypic plasticity can set the baseline for sympatric speciation and evolutionary divergence of habitat morphs (see Agrawal 2001). In fact, environmental stress gradients in coastal intertidal habitats related to heat, desiccation, salinity, and wave action can provide the adequate setting for adaptive processes in patellids (Branch 1981). If reproductive isolation is enforced by the ecological characteristics of each habitat, then biological separated species can be revealed. A good example comes from the diversification in *Nacella* limpets in the Magellanic Province (South America) (González-Wevar *et al.* 2011). Ecological speciation and restricting levels of gene flow resulting from ecologically based divergent selection are considered the main driving processes of such diversification. The possibility of speciation along ecological gradients, without the need of a complete allopatric isolation, has also been shown for the Hawaiian endemic limpets of the genus *Cellana* (Bird *et al.* 2011).

4.2. Population genetic structure and contemporary connectivity

Genetic differentiation estimates among archipelagos revealed a highly structured pattern in the *P. candei* complex across Macaronesia. Populations of *P. candei* from Azores are the most isolated and exhibit the highest level of differentiation from the remaining archipelagos. To a lesser extent, populations from Madeira and Canaries, which are about 400 km apart, also show significant genetic differentiation and limited contemporary connectivity. In fact, migratory events between Madeira and Canaries are unlikely, if not entirely absent, despite the fact that these archipelagos are geographically closer to each other. Only three individuals showed a very slightly probability of being migrants between these two archipelagos. Selvagens islands, which stand at approximately two-thirds of the way between Madeira and the Canary Islands, and could act as a putative stepping stone for gene flow requires further examination. As for Azores, despite the wide geographical distribution of its islands across (~ 600 km), the minimum and maximum distances among any pair of adjacent islands that pelagic larvae must travel among islands are approximately 32 and 220 km, respectively. Such distances do not seem to offer an obstacle and allow populations' gene pool across all islands to be homogenized via larval transport. Although genetic differentiation among archipelagos seems to be highly correlated with geographic distance, the unbalanced nature of sampling, the fact that connectivity within archipelago (i.e. Azores) does not follow IBD, and the results provided by

BARRIER (see Fig. 7) and BIMr analyses, suggest that the most likely barriers to gene flow in *P. candei* across the Macaronesia archipelagos are also associated to historical and contemporary limitations imposed by the masses of water that separate them, and are not a direct result of the geographical distance per se. In this case, the historical shifting of ocean circulation processes and the current oceanographic complexity and mesoscale variability across Macaronesia, with meanders, high eddy kinetic energy, upwellings, and several masses with distinct thermohaline characteristics (see Johnson and Stevens 2000; Alves *et al.* 2002; Rogerson *et al.* 2004), may have acted as a strong physical barriers to gene exchange among archipelagos. Such barriers to gene flow, however, cannot fully exclude limitative dispersal across space or IBD pattern in *P. candei* throughout Macaronesia. Not only larval mortality rates increase almost exponentially as they move away from the coast into offshore waters (Cowen *et al.* 2000), but the PLD of *P. candei* may also be shorter than expected, or of a narrower range of what is generally referred for other patellids (Ribeiro 2008). Under such scenario, because larvae are less likely to travel longer distances, populations farther away from each other would be more genetically distinct.

The failure of some microsatellites, which were isolated from the genome of *P. candei* samples from Azores, in amplifying individuals from Madeira and Canaries may further suggest substantial genetic break among archipelagos. The reduced marker polymorphism in southern samples may reflect pronounced sequence differences among subspecies due to their genetic divergence, thus entailing an upward ascertainment bias as markers were developed from Azorean morphotypes. This may explain the contrasting results of this study and those provided by Sá-Pinto *et al.* (2005, 2008). In their study, samples from Azores and Madeira grouped together in a well-supported clade, leaving Canaries more distant related. According to the same authors, the scenario of a single colonization event for each archipelago associated with the absence of historical gene flow between them is the most likely. The direction and timing of such colonization events is still unclear, and methods such as the approximate Bayesian computation (Beaumont *et al.* 2002) may be useful in contrasting demographic hypothesis about the evolutionary history of limpets in Macaronesia, provided that all archipelagos are sampled (including Selvagens). Even so, it seems plausible to accept that upon a single event of colonization, limpet populations in each archipelago remained isolated and evolved/adapted allopatrically to the environmental specificities of each archipelago. It is thought that *P. candei candei* from the Selvagens islands is the ancestral species that first colonized the Canaries and Madeira and only later the Azores (Weber and Hawkins 2002; Sá-Pinto *et al.* 2008). This evolutionary pattern tracks each archipelago's time of origin with Selvagens being the oldest (~ 29.5 Ma) and Azores the youngest (< 6 Ma), but the exact sequence of colonization is still unresolved. Changes in sea level and ocean circulation associated with major historical episodes such as the tectonic closure of the Isthmus of Panama (Haug and Tiedemann 1998), the Plio-Pleistocene glacial cycles (Maggs *et al.* 2008), and the closing off of the western basin of the Mediterranean from the Atlantic (Krijgsman *et al.* 1999) may have contributed to the expansion and allopatric differentiation of *P. candei* throughout Macaronesia. As suggested for the endemic Macaronesia periwinkle *Tectarius striatus* (den Broeck *et al.* 2008), *P. candei* may have colonized Macaronesia in periods when sea levels were lower, so that seamounts peaked above sea level and acted as stepping stones between

archipelagos. Therefore, adaptive processes associated with niche differentiation and physical/geographical isolation among populations may correspond to the underlying mechanisms for *P. candei* diversification in Macaronesia. In the absence of gene flow between populations, reproductive isolation would arise gradually as a result of mutation, genetic drift and natural selection driven by differences in local environmental conditions (Hoskins *et al.* 2005). In fact, allopatric speciation under restrictive gene flow is believed to be one of the most common modes of speciation in nature (Schluter 2009). Yet, providing that reproductive isolation is complete, secondary events of colonization followed by the weakening of physical/ geographical barriers are not to be excluded and, for instance, may have contributed to the coexistence of *P. candei candei* (the Selvagens ecotype) and *P. candei crenata* in Canaries islands; although isolated specimens of *P. candei candei* were identified in El Hierro and Tenerife islands, this co-occurrence is now mainly restricted to a single island: Fuerteventura (González-Lorenzo *et al.* 2016).

4.3. Conservation of limpet population in Macaronesia

The aim of conservation is not simply to safeguard species from going extinct, but also to guarantee that morphological and genetic variation in natural populations is preserved. Efforts toward ensuring the conservation of limpet populations in Macaronesia are highly recommended, especially considering that their low genetic diversity and lack of gene exchange between archipelagos suggest they may be highly vulnerable. Taking into account its endemic nature and the negative impact of over-exploitation in coastal communities, the risk of complete extinction of *P. candei* in Macaronesia is therefore conceivable. Yet, because our study failed to detect the occurrence of population bottlenecks, which would be expected under the known demographic decline of *P. candei* in Macaronesia, the effective population sizes must be still large enough to prevent critical losses to genetic variability (see Pujolar *et al.* 2011). However, when comparing to unexploited populations of patellids elsewhere (e.g. Perez *et al.* 2007; Ribeiro *et al.* 2010), genetic diversity in the *P. candei* complex is fairly low. Despite the challenges associated with such comparisons, especially because different microsatellite loci may generate different levels of variation, the reduced genetic diversity observed can be a consequence of high levels of population inbreeding caused by intensive exploitation. As harvesting is mainly aimed at larger individuals (Martins *et al.* 2008), the more fertile individuals with higher reproductive outputs are likely less abundant than expected. This may lead to severe evolutionary and ecological consequences for the biology, life-history traits, and survival of the species (Fenberg and Roy 2008).

Our results support the view that populations from each archipelago should be managed for conservation as distinct units. Within the *P. candei* complex, *P. candei candei* is considered in danger of extinction under the Spanish Catalogue of Endangered Species and is now mainly restricted to one single island: Fuerteventura (Canary Islands). This is likely a consequence of overexploitation (Núñez *et al.* 2003), but selective and evolutionary-related processes may have also been involved (González-Lorenzo *et al.* 2016). Recently, a forced ban to its capture and a conservational plan has been put in place by regional authorities. Aside from Fuerteventura, *P. candei candei* occurs more abundantly in

the Selvagens, uninhabitable islands that are protected under the Portuguese status of Nature Reserve. As for the remaining limpets, each Regional authority has established protective measures and rules for their harvesting. Legislation does not differ much among regions, with the establishment of limpet no-take areas, minimum legal catch sizes, and seasonal fishing closures. Unfortunately, these actions have been largely ineffective in protecting such resource (Martins *et al.* 2011; López *et al.* 2012; Diogo *et al.* 2016; Riera *et al.* 2016), mostly because of illegal harvesting and lack of or insufficient enforcement. Protective measures need to be adjusted to the particular life-history traits of *P. candei* in each archipelago (e.g. temporal variation in reproduction, recruitment, population dynamics). Furthermore, both environmental awareness programs to general population and coastal enforcement by local authorities should be stimulated. Above all, we suggest the establishment of fully enforced closed zones to limit the access to rocky shore poachers and allow limpet populations to grow in numbers and individual sizes. These off-limit areas should be regularly surveyed and take into consideration population connectivity patterns within archipelagos. Our data suggest that, at least for Azores, such areas should distance no more than ~ 200 km, to allow proper gene exchange between populations. Additional sampling throughout all Macaronesia islands can further sharpen our knowledge about connectivity patterns in *P. candei* and help better defining the establishment of such areas within each archipelago.

Under the very probable assumption that *P. candei* from each archipelago forms a geographically and/or ecologically isolated population, the various subspecies within the *P. candei* complex as previously proposed by Christiaens (1973) may be best thought as true species using the denomination: *Patella candei* in Selvagens, *Patella gomesii* in Azores, *Patella ordinaria* in Madeira, and *Patella crenata* for Canaries. Whether this would facilitate current taxonomic misinterpretations and conservation needs, further research is still required, especially in diagnosing intrinsic reproductive isolation (Frankham *et al.* 2012). Ultimately, this elevation of subspecies to species level would be in agreement with stock delimitation and units of conservation (Hawkins *et al.* 2016) with potential benefits for management plans aimed at the preservation of limpets stocks across the Macaronesia region.

Acknowledgments

We sincerely thank two anonymous reviewers for their useful comments that improved significantly this manuscript. We thank Manuel Rivas and Manuel Enes for helping with DNA extractions and morphometric measurements, respectively. Field and sampling assistance was provided by Maria Vale, Afonso Prestes, Joana Pombo in Santa Maria (Azores), José Azevedo in Pico (Azores), André Amaral in Terceira (Azores), Pedro Raposeiro in Flores and Corvo (Azores), and Fernando Tuya and Manuel Rivas in Canarias. We thank Michael Collyer (Western Kentucky University) for help in geometric morphometrics. This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE—Operational Competitiveness Programme and national funds through FCT—Foundation for Science and Technology, under the projects PTDC/BIA-BIC/115837/2009 and PEst-C/MAR/ LA0015/2013, by the Strategic Funding UID/Multi/04423/2013

through national funds provided by FCT—Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020 and by cE3c funding (Ref: UID/BIA/00329/2013). JF was funded by a PhD grant M3.1.2/ F/021/2011 by the Regional Government of the Azores. GMM was supported by postdoctoral grants awarded by FCT, Portugal (SFRH/ BDP/63040/2009). PAR was funded by the Portuguese Foundation for Science and Technology, through a postdoctoral grant ref. SFRH/BPD/69232/2010 funded through QREN and COMPETE, and the strategic project UID/MAR/04292/2013 granted to MARE.

References

Adams DC, Rohlf FJ, Slice DE (2013) A field comes of age: geometric morphometrics in the 21st century. *Hystrix* 24: 7-14.

Adams DC, Collyer M, Kaliontzopoulou A, Sherratt E (2016) geomorph: Geometric morphometric analyses of 2D/3D landmark data 3.0.2. Available from: https://cran.r-project.org/package=geomorph.

Adams DC, Otárola-Castillo E (2013) geomorph: an R package for the collection and analysis of geometric morphometric shape data. *Methods in Ecology and Evolution* 4: 393-399.

Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. *Science* 294: 321-326.

Alves M, Gaillard F, Sparrow M, Knoll M, Giraud S (2002) Circulation patterns and transport of the Azores Front-Current system. *Deep Sea Research Part II: Topical Studies in Oceanography* 49: 3983-4002.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26: 32-46.

Anderson MJ, Gorley RN, Clarke KR (2008) *PERMANOVA for PRIMER: guide to software and statistical methods*. PRIMER-E Ltd., Plymouth, United Kingdom.

Ávila S, Melo C Berning B, Cordeiro R Landau B, da Silva CM (2016) *Persististrombus coronatus* (Mollusca: Strombidae) in the lower Pliocene of Santa Maria Island (Azores, NE Atlantic): Paleoecology, paleoclimatology and paleobiogeographic implications. *Palaeogeography, Palaeoclimatology, Palaeoecology* 441: 912-923.

Barber PH, Palumbi SR, Erdmann MV, Moosa MK (2000) A marine Wallace's line?. *Nature* 406: 692-693.

Baylac M, Villemant C, Simbolotti G (2003) Combining geometric morphometrics with pattern recognition for the investigation of species complexes. *Biological Journal of the Linnean Society* 80: 89-98.

Beaumont MA, Zhang W, Balding DJ (2002) Approximate Bayesian Computation in population genetics. *Genetics* 162: 2025-2035.

Bird CE, Holland BS, Bowen BW, Toonen RJ (2011) Diversification of sympatric broadcast-spawning limpets (*Cellana* spp.) within the Hawaiian archipelago. *Molecular Ecology* 20: 2128-2141.

Boaventura D, Alexander M, Santina PD, Smith ND, Ré P, Fonseca LC, Hawkins SJ (2002) The effects of grazing on the distribution and composition of low-shore algal communities on the central coast of Portugal and on the southern coast of Britain. *Journal of Experimental Marine Ecology and Biology 2*67: 185-206.

Bookstein FL (1991) *Morphometric tools for landmark data: geometry and biology*. Cambridge University Press, New York.

Branch GM (1981) The biology of limpets: physical factors, energy flow, and ecological interactions. *Oceanography and Marine Biology: an Annual Review* 19: 235-380.

Branch GM, Marsh AC (1978) Tenacity and shell shape in six *Patella* species: adaptive features. *Journal of Experimental Marine Biology and Ecology* 3: 111-130.

Cabral JP (2007) Shape and growth in European Atlantic *Patella* limpets (Gastropoda, Mollusca). Ecological implications for survival. *Web Ecology* 7: 11-21.

Carlsson J (2008) Effects of microsatellite null alleles on assignment testing. *Journal of Heredity* 99: 616-623.

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621-631.

Christiaens J (1973) Révision du genre *Patella* (Mollusca, Gastropoda). *Bulletin du Muséum National D'histoire Naturelle* 182: 1305-1392.

Coleman RA, Underwood AJ, Benedetti-Cecchi, Aberg P, Arenas F, Arrontes J, Castro J, Hartnoll RG, Jenkins SR, Paula J, Santina PD, Hawkins SJ (2006) A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia* 147: 556-564.

Collyer ML, Sekora DJ, Adams DC (2015) A method for analysis of phenotypic change for phenotypes described by high-dimensional data. *Heredity* 115: 357-365.

Côrte-Real HBSM, Hawkins SJ, Thorpe JP (1996) Population differentiation and taxonomic status of the exploited limpet *Patella candei* in the Macaronesian Islands (Azores, Madeira, Canaries). *Marine Biology* 125: 141-152.

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of marine populations: open or closed?. *Science* 287: 857-859.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review of Marine Science* 1: 443-466.

Davis MA, Douglas MR, Collyer ML, Douglas ME (2016) Deconstructing a species-complex: geometric morphometric and molecular analyses define species in the western rattlesnake (*Crotalus viridis*). *PLoS ONE* 11: e0146166.

den Broeck HV, Breugelmans K, Wolf De H, Backeljau T (2008) Completely disjunct mitochondrial DNA haplotype distribution without a phylogeographic break in a planktonic developing gastropod. *Marine Biology* 153: 421-429.

Denny MW (2000) Limits to optimization: fluid dynamics, adhesive strength and the evolution of shape in limpet shells. *Journal of Experimental Biology* 203: 2603-2622.

Diogo H, Pereira JG, Schmiing M (2016) Catch me if you can: Non-compliance of limpet protection in the Azores. *Marine Policy* 63: 92-99.

Dodd JM (1957) Artificial fertilisation, larval development and metamorphosis in *Patella vulgata* L. and *Patella caerulea* L. *Pubblicazioni della Stazione Zoologica di Napoli* 29: 172-185.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetic Resources* 4: 359-361.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611-2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Faria J, Froufe E, Tuya F, Alexandrino P, Pérez-Losada M (2013) Panmixia in the endangered slipper lobster *Scyllarides latus* from the Northeastern Atlantic and Western Mediterranean. *Journal of Crustacean Biology* 33: 557-566.

Faria, J, Pita A, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Neto AI, Presa P (2016) A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 775-781.

Faubet P, Gaggiotti OE (2008) A new Bayesian method to identify the environmental factors that influence recent migration. *Genetics* 178: 1491-1504.

Faubet P, Waples RS, Gaggiotti OE (2007) Evaluating the performance of a multilocus Bayesian method for the estimation of migration rates. *Molecular Ecology* 16: 1149-1166.

Fenberg PB, Roy K (2008) Ecological and evolutionary consequences of size-selective harvesting: how much do we know?. *Molecular Ecology* 17: 209-220.

Frankham R, Ballou JD, Dudash MR, Eldrigde MDB, Fenster CB, Lacy RC, Mendelson III JR, Porton IJ, Ralls K, Ryder OA (2012) Implications of different species concepts for conserving biodiversity. *Biological Conservation* 153: 25-31.

Gerlach G, Jueterbock A, Kraemer P, Deppermann J, Harmand P (2010) Calculations of population differentiation based on $G_{(ST)}$ and D: forget $G_{(ST)}$ but not all of statistics! *Molecular Ecology* 19: 3845-3852.

González-Lorenzo G, Hernández EM, Pérez-Dionis G, Hernández AB, Santos BG, Diez JB (2016) Ineffective conservation threatens *Patella candei*, an endangered limpet endemic to the Macaronesian islands. *Biological Conservation* 192: 428-435.

González-Wevar CA, Nakano T, Cañete JI, Poulin E (2011) Concerted genetic, morphological and ecological diversification in Nacella limpets in the Magellanic Province. *Molecular Ecology* 20: 1936-1951.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity* 86: 485-486.

Guerra-Varela J, Colson I, Backeljau T, Breugelmans K, Hughes RN, Rolán-Alvarez E (2009) The evolutionary mechanism maintaining shell shape and molecular differentiation between two ecotypes of the dogwhelk *Nucella lapillus*. *Evolutionary Ecology* 23: 261-280.

Guichoux E, Lagache L, Wagner S, Chaumeil P, Leger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11: 591-611.

Gunz P, Mitteroecke P (2013) Semilandmarks: a method for quantifying curves and surfaces. *Hystrix, the Italian Journal of Mammalogy* 24: 103-109.

Harley CDG, Denny MW, Mach KJ, Miller LP (2009) Thermal stress and morphological adaptations in limpets. *Functional Ecology* 23: 292-301.

Haug GH, Tiedemann R (1998) Effect of the formation of the Isthmus of Panama on Atlantic Ocean thermohaline circulation. *Nature* 393: 673-678.

Hawkins SJ, Côrte-Real HBSM, Martins HR, Santos RS, Martins AMF (1990) A note on the identity of *Patella* in the Azores. *Açoreana* (Suppl.): 167-173.

Hawkins SJ, Côrte-Real HBSM, Pannacciulli FG, Weber LC, Bishop JDD (2000) Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. *Hydrobiologia* 440: 3-17.

Hawkins SJ, Bohn K, Sima DW, Ribeiro P, Faria J, Presa P, Pita A, Martins GM, Neto AI, Burrows MT, Genner MJ (2016) Fisheries stocks from an ecological perspective: Disentangling ecological connectivity from genetic interchange. *Fisheries Research* 179: 333-341.

Hawkins SJ, Hartnoll RG (1983) Grazing of intertidal algae by marine invertebrates. *Oceanography and Marine Biology: an Annual Review* 21: 195-282.

Hoskins CJ, Higgie M, McDonald KR, Moritz C (2005) Reinforcement drives rapid allopatric speciation. *Nature* 437: 1353-1356.

Johnson J, Stevens I (2000) A fine resolution model of the eastern North Atlantic between the Azores, the Canary Islands and the Gibraltar Strait. *Deep-Sea Research I* 47: 875-899.

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24: 1403-1405.

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* 11: 94.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. *Molecular Ecology* 17: 4015-4026.

Keever CC, Sunday J, Puritz JB, Addison JA, Toonen RJ, Grosberg RK, Hart MW (2009) Discordant distribution of populations and genetic variation in a sea star with high dispersal potential. *Evolution* 63: 3214-3227.

Kelly RP, Palumbi SR (2010) Genetic structure among 50 species of the Northeastern Pacific rocky intertidal community. *PLoS ONE* 5: e8594.

Krijgsman W, Hilgen FJ, Raffi I, Sierra FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature* 400: 652-655.

Lande R (1988) Genetics and demography in biological conservation. *Science* 241: 1455-1460.

López C, Poladura A, Hernández JC, Martín L, Conceptíon L, Sangil C, Clemente S (2012) Contrasting effects of protection from harvesting in populations of two limpet species in a recently established marine protected area. *Scientia Marina* 76: 799-807.

Lowell RB (1986) Crab predation on limpets: predator behavior and defensive features of the shell morphology of the prey. *Biological Bulletin* 171: 577-596.

Maggs CA, Castilho R, Foltz D, Henzler C, Jolly MT, Kelly J, Olsen J, Perez KE, Stam W, Vänölä R, Viard F, Wares J (2008) Evaluating signatures of the glacial refugia for North Atlantic benthic marine taxa. *Ecology* 89: 108-122.

Manni F, Guérard E, Heyer E (2004) Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by using Monmonier's algorithm. *Human Biology* 76: 173-190

Martins HR, Santos RS, Hawkins SJ (1987) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Thompson RC, Neto AI, Hawkins SJ, Jenkins SR (2010) Exploitation of intertidal grazers as a driver of community divergence. *Journal of Applied Ecology* 47: 1282-1289.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Medeiros AR, Thompson RC (2011) Illegal harvesting affects the success of fishing closure areas. *Journal of the Marine Biological Association UK* 91: 929-937.

Martins GM, Borges CDG, Vale M, Ferraz RR, Martins HR, Santos RS, Hawkins SJ (2017) Exploitation promotes earlier sex change in a protandrous patellid limpet, *Patella aspera* Röding, 1798. *Ecology and Evolution* 7(10): 3616-3622.

McKay JK, Latta RG (2002) Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution* 17: 285-291.

Meirmans PG (2014) Non-convergence in Bayesian estimation of migration rates. *Molecular Ecology Resources* 14: 726-733.

Núñez J, Brito MC, Riera R, Docoito JR, Monterroso O (2003) Distribución actual de las poblaciones de *Patella candei* d'Orbigny, 1840 (Mollusca, Gastropoda) en las islas Canarias - Una especie en peligro de extinción. *Boletin Instituto Espanol de Oceanografia* 19: 371-377.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537-2539.

Perez M, Branco M, Llavona A, Ribeiro PA, Santos AM, Hawkins SJ, Dávila JA, Presa P, Alexandrino P (2007) Development of microsatellite loci for the black-footed limpet, *Patella depressa*, and cross-amplification in two other *Patella* species. *Conservation Genetics* 8: 739-742.

Portnoy DS, Hollenbeck CM, Belcher CN, Driggers III WB, Frazier BS, Gelsleichter J, Grubbs RD, Gold JR (2014) Contemporary population structure and post-glacial genetic demography in a migratory marine species, the blacknose shark, *Carcharhinus acronotus*. *Molecular Ecology* 23: 5480-5495.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Pujolar JM, Bevacqua D, Capoccioni F, Ciccoti E, De Leo GA, Zane L (2011) No apparent genetic bottleneck in the demographically declining European eel using molecular genetics and forward-time simulations. *Conservation Genetics* 12: 813-825.

R Core Team (2014) R: *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/.

Raymond M, Rousset F (1995) GENEPOP (version 1.2) - population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Ribeiro PA, Branco M, Hawkins SJ, Santos AM (2010) Recent changes in the distribution of a marine gastropod, *Patella rustica*, across the Iberian Atlantic coast did not result in diminished genetic diversity or increased connectivity. *Journal of Biogeography* 37(9): 1782-1796.

Riera R, Pérez Ó, Álvarez O, Simón D, Díaz D, Monterroso, Núnez J (2016) Clear regression of harvested intertidal mollusks. A 20-year (1994-2014) comparative study. *Marine Environmental Research* 113: 56-61.

Rogerson M, Rohling EG, Weaver PPE, Murray JW (2004) The Azores Front since the Last Glacial Maximum. *Earth and Planetary Science Letters* 222: 779-789.

Rohlf FJ (2015) The tps series of software. *Hystrix, the Italian Journal of Mammalogy* 26: 1-4.

Rohlf FJ, Slice D (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* 39: 40-59.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* 145: 1219-1228.

Ruane S (2015) Using geometric morphometrics for integrative taxonomy: an examination of head shapes of milksnakes (genus *Lampropeltis*). *Zoological Journal of the Linnean Society* 174: 394-413.

Sá-Pinto A, Branco MS, Harris DJ, Alexandrino P (2005) Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. *Journal of Experimental Marine Biology and Ecology* 325: 95-110.

Sá-Pinto A, Branco A, Sayanda D, Alexandrino P (2008) Patterns of colonization, evolution and gene flow in species of the genus *Patella* in the Macaronesian Islands. *Molecular Ecology* 17: 519-532.

Sandoval-Castillo J, Beheregaray LB (2015) Metapopulation structure informs conservation management in a heavily exploited coastal shark (*Mustelus henlei*). *Marine Ecology Progress Series* 533: 191-203.

Santos RS, Hawkins SJ, Monteiro LR, Alves M, Isidro EJ (1995) Marine research, resources and conservation in the Azores. *Aquatic Conservation: Marine and Freshwater Ecosystems* 5: 311-354.

Schluter D (2009) Evidence for ecological speciation and its alternative. *Science* 323: 737-741.

Sokolova IM, Berger VJ (2000) Physiological variation related to shell colour polymorphism in White Sea *Littorina saxatilis*. *Journal of Experimental Marine Biology and Ecology* 245: 1-23.

Szpiech ZA, Jakobsson M, Rosenberg NA (2008) ADZE: a rarefaction approach for counting alleles private to combinations of populations. *Bioinformatics* 27: 2498-2504.

Trussell GC (2000) Phenotypic clines, plasticity, and morphological trade-offs in an intertidal snail. *Evolution* 54: 151-66.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535-538.

Verhoeven KJF, Simonsen KL, Mcintyre LM (2005) Implementing false discovery rate control: increasing your power. *Oikos* 3: 643-647.

Villegas J, Feliciangeli MD, Dujardin JP (2002) Wing shape divergence between *Rhodnius prolixus* from Cojedes (Venezuela) and *R. robustus* from Mérida (Venezuela). *Infection, Genetics and Evolution* 2: 121-128.

Weber LI, Hawkins SJ (2002) Evolution of the limpet *Patella candei* d'Orbigny (Mollusca: Patellidae) in Atlantic archipelagos: human intervention and natural processes. *Biological Journal of the Linnean Society* 77: 341-353.

White TA, Fotherby HA, Stephens PA, Hoelzel AR (2011) Genetic panmixia and demographic dependence across the North Atlantic in the deep-sea fish, blue hake (*Antimora rostrata*). *Heredity* 106: 690-699.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* 163: 1177-1191.

Wolf H, Backeljau T, Medeiros R, Verhagen R (1997) Microgeographical shell variation in *Littorina striata*, a planktonic developing periwinkle. *Marine Biology* 129: 331-342.

Wright S (1943) Isolation by distance. *Genetics* 28: 114-138.

Zelditch ML, Swiderski DL, Sheets HD (2012) *Geometric morphometrics for biologists: a primer*. 2nd Edition. Elsevier Academic Press, London.

SUPPLEMENTARY MATERIAL

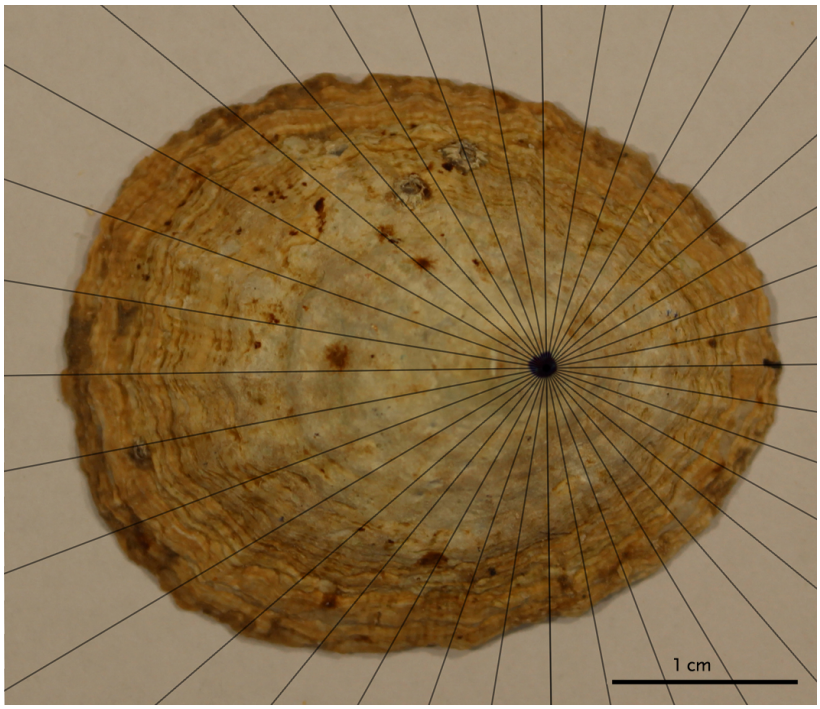


Figure S1. Representative imaging of a *Patella candei* shell used for geometric morphometrics. Shells were oriented on a fan by superimposed the anterior and posterior ends along the horizontal line of the fan. The apex was made to coincide with the vertical line of the fan and all shells were placed in the fan with their anterior end facing the right side. The fan consisted of a set of 18 lines angularly distanced by 10º increments.

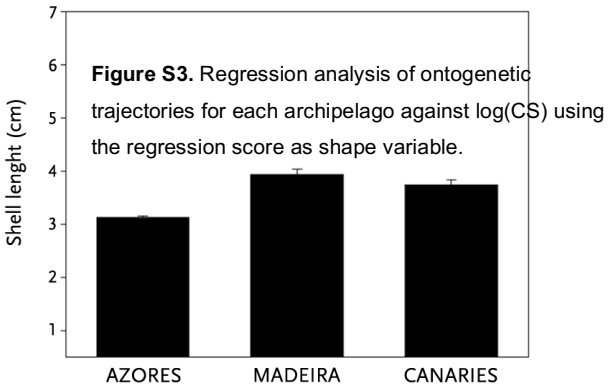


Figure S3. Regression analysis of ontogenetic trajectories for each archipelago against log(CS) using the regression score as shape variable.

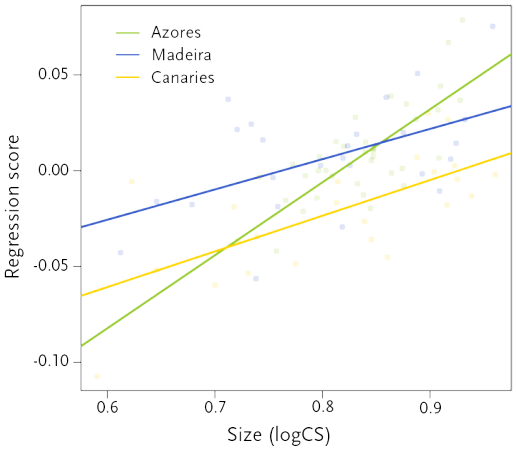


Figure S2. Mean (+SE) *Patella candei* shell length across archipelagos.

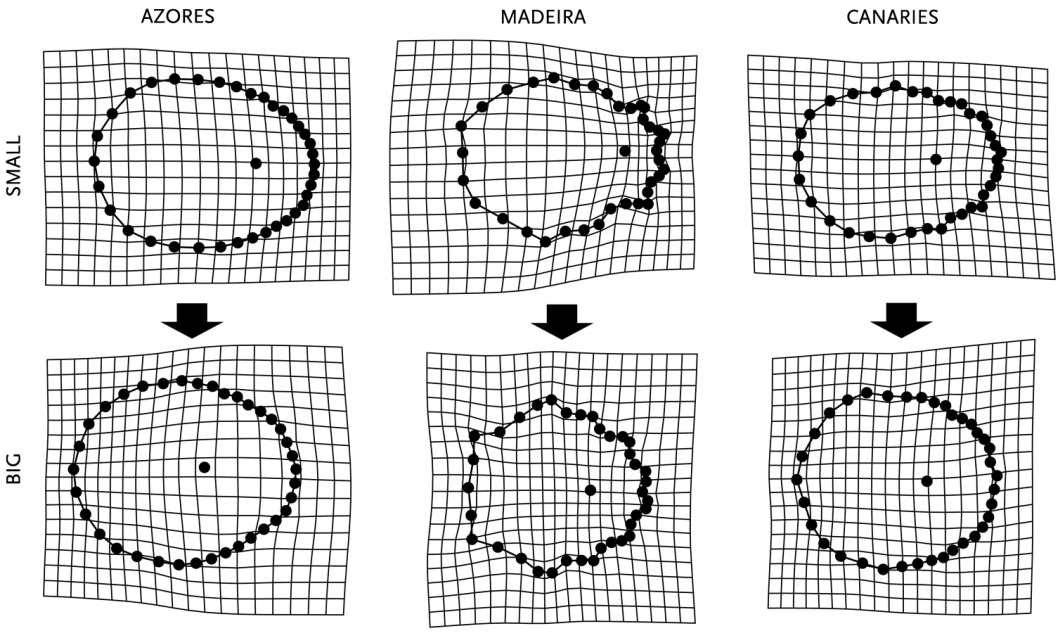


Figure S4. Deformation grids for shell shape variation of the three smaller and bigger limpets of each archipelago.

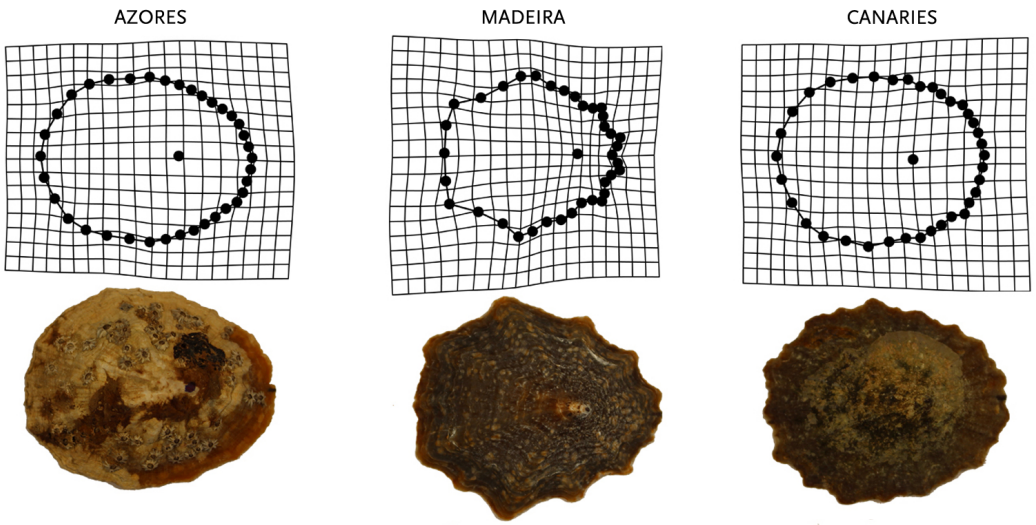


Figure S5. Individual shells of specimens identified as more similar/closest to each group mean shape. Thin-plate spline deformations grids are shown for each archipelago shape variation against overall mean shape.

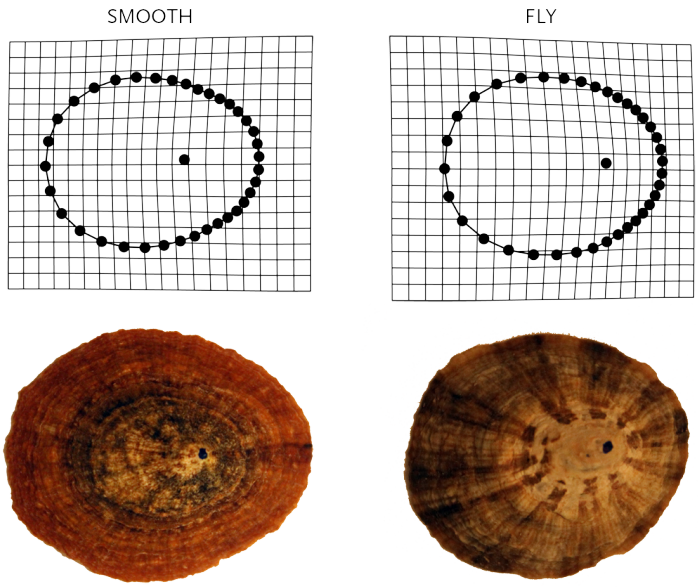


Figure S6. Individual shells of specimens identified as more similar/closest to each group mean shape. Thin-plate spline deformations grids are shown for each morphotypes ('smooth' and 'fly') shape variation against overall mean shape.

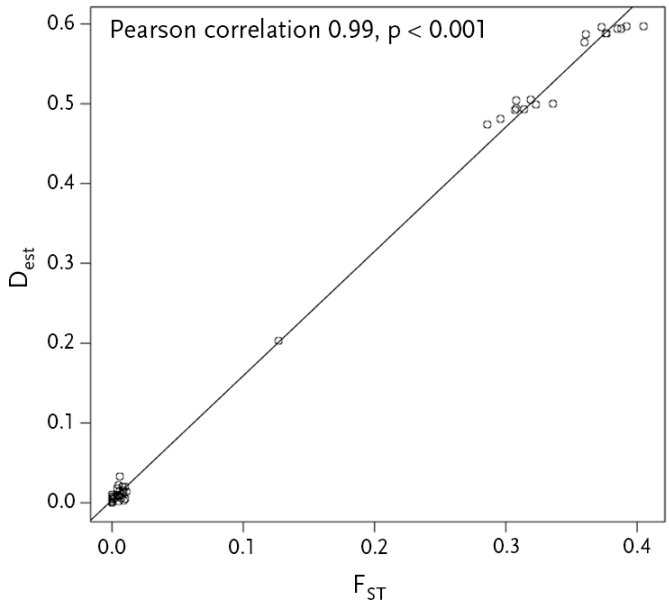


Figure S7. Correlation of pairwise estimates of F_{ST} and D_{est} between populations of *Patella candei* in NE Atlantic.

Table S1. A two-way PERMANOVA examining differences in shell length within archipelagos and islands.

	df	MS	F
ARCHIPELAGO	2	40.259	1.2015
ISLAND (ARCHIPELAGO)	9	43.914	127.09***
Residual	905	0.34554	
Total	916		

*** P < 0.001

Table S2. Correlation matrices between *P. candei* shell morphological variables.

	Distance measures					Morphometric descriptors			
	SL	SW	SWA	SAA	SH	BE	BEC	CO	CE
Shell length (SL)	1.00								
Shell width (SW)	0.99	1.00							
Shell width at apex (SWA)	0.98	0.99	1.00						
Distance from apex to anterior tip (SAA)	0.93	0.91	0.94	1.00					
Shell height (SH)	0.77	0.77	0.82	0.91	1.00				
Base ellipticity (BE)	0.39	0.53	0.49	0.36	0.36	1.00			
Base eccentricity (BEC)	0.08	0.03	0.19	0.26	0.36	-0.19	1.00		
Conicity (CO)	-0.08	-0.06	0.02	0.22	0.55	0.12	0.46	1.00	
Cone eccentricity (CE)	0.26	0.26	0.33	0.59	0.68	0.18	0.46	0.75	1.00

Table S3. A two-way PERMANOVA examining differences in shell conicity among archipelagos and islands.

	df	MS	F	Components of variation (%)
ARCHIPELAGO	2	0.4294	7.3934*	53
ISLAND (ARCHIPELAGO)	9	7.5844E-2	51.737***	18
Residual	905	1.4659E-3		29
Total	916			
Pairwise comparisons				
AZORES = CANARIES; AZORES ≠ MADEIRA; CANARIES ≠ MADEIRA				

* P < 0.05, *** P < 0.001

Table S4. A two-way PERMANOVA examining differences in shell base ellipticity among archipelagos and islands. Individual shell length was used as the covariate.

	df	MS	F	Components of variation (%)
SL	1	0.27965	25.097***	12
ARCHIPELAGO	2	7.2687E-2	5.0872*	19
ISLAND (ARCHIPELAGO)	9	1.7081E-2	12.087***	9
SL × ARCHIPELAGO	2	2.9074E-3	2.0574	1
SL × ISLAND (ARCHIPELAGO)	9	2.2428E-3	1.5871	1
Residual	893	1.4131E-3		58
Total	916			
Pairwise comparisons				
AZORES = CANARIES; AZORES ≠ MADEIRA; CANARIES ≠ MADEIRA				

* P < 0.05, *** P < 0.001

Table S5. Procrustes ANOVA examining differences in patterns of shell shape variation among archipelagos (10 000 random permutations) for SMALL and BIG datasets. Centroid size (CS) was used as a covariate.

Small limpets	df	MS	F
SIZE *log(CS)*	1	0.0044	1.630
ARCHIPELAGO	2	0.0083	3.055*
SIZE *log(CS)* × ARCHIPELAGO	2	0.0029	1.064
Total	29		
Pairwise comparisons			
AZORES = CANARIES; AZORES ≠ MADEIRA; CANARIES ≠ MADEIRA			

Big limpets	df	MS	F
SIZE *log(CS)*	1	0.0014	0.7670
ARCHIPELAGO	2	0.0093	5.0378**
SIZE *log(CS)* × ARCHIPELAGO	2	0.0024	1.2970
Total	23		
Pairwise comparisons			
AZORES = CANARIES; AZORES ≠ MADEIRA; CANARIES ≠ MADEIRA			

* P < 0.05, ** P < 0.01

Table S6. Procrustes ANOVA examining differences in patterns of shell shape variation among *P. candei* morphotypes in Azores (10 000 random permutations). Centroid size (CS) was used as a covariate. Slope pairwise comparisons among morphotypes are shown; contrasts in slope vector length and angles between slope vectors are shown in upper and lower diagonal, respectively.

	df	R^2	Z	F
SIZE *log(CS)*	1	0.281	11.185	26.605***
MORPH	1	0.081	4.309	7.686**
SIZE *log(CS)* × MORPH	1	0.077	4.474	7.305**
Total	56			
Slope pairwise comparison				
	FLY		SMOOTH	
FLY	-		4.334 **	
SMOOTH	140.7		-	

** P < 0.01, *** P < 0.001; Effect sizes (Z) are standard deviations of observed.

Table S7. Genetic variation observed at twelve microsatellite loci within eleven populations sampled for *Patella candei* (see Fig. 1 for population codes).

	FLO	COR	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
N	58	49	54	54	46	49	53	49	50	50	48	560
CAN9												
N_A	9	10	11	10	10	9	10	9	10	0	0	12
$A_R(30)$	7.16	8.03	8.42	7.59	8.41	7.74	7.43	7.16	7.96	-	-	-
$A_P(30)$	0.17	0.22	0.32	0.19	0.17	0.00	0.08	0.00	0.15	-	-	-
H_O	0.672	0.755	0.704	0.704	0.739	0.830	0.774	0.694	0.755	0.000	0.000	0.602
H_E	0.808	0.832	0.856	0.816	0.843	0.834	0.783	0.817	0.792	0.000	0.000	0.671
F_{IS}	**0.169**	0.093	**0.180**	**0.139**	**0.124**	0.005	0.012	0.152	0.048	-	-	**0.105**
Null	0.07[a]	0.03	0.09[a]	0.06	0.06	0.01	0.02	0.06[a]	0.02	-	-	-
CAN18												
N_A	18	14	18	13	12	14	14	16	16	5	5	22
$A_R(30)$	11.04	10.02	11.34	9.22	9.82	9.78	9.44	11.15	10.58	4.75	3.98	-
$A_P(30)$	0.59	0.12	0.59	0.35	0.01	0.44	0.02	0.12	0.41	0.48	0.00	-
H_O	0.537	0.510	0.520	0.469	0.543	0.511	0.462	0.500	0.600	0.242	0.577	0.497
H_E	0.841	0.871	0.888	0.837	0.882	0.860	0.849	0.872	0.866	0.666	0.546	0.816
F_{IS}	**0.363**	**0.417**	**0.417**	**0.442**	**0.386**	**0.409**	**0.459**	**0.429**	**0.310**	**0.640**	**-0.058**	**0.401**
Null	0.16[a]	0.19[a]	0.19[a]	0.19[a]	0.18[a]	0.19[a]	0.21[a]	0.19[a]	0.14[a]	0.26[a]	0.04[a]	
CAN23												
N_A	4	3	5	4	5	3	4	3	2	3	2	8
$A_R(30)$	2.52	2.52	3.18	2.76	3.35	2.31	2.78	2.67	2.00	2.20	1.78	-
$A_P(30)$	0.18	0.00	0.33	0.14	0.41	0.00	0.22	0.00	0.00	1.20	0.00	-
H_O	0.448	0.347	0.556	0.426	0.500	0.396	0.481	0.367	0.360	0.143	0.083	0.497
H_E	0.435	0.394	0.462	0.452	0.476	0.432	0.430	0.408	0.347	0.135	0.081	0.368
F_{IS}	-0.031	0.120	-0.206	0.058	-0.051	0.085	-0.119	0.101	-0.039	-0.057	-0.033	-0.016
Null	0.00	0.04	0.00	0.01	0.00	0.02	0.00	0.03	0.00	0.00	0.00	
CAN25												
N_A	6	6	9	8	7	7	7	7	8	5	5	14
$A_R(30)$	4.96	5.06	6.94	6.55	5.97	6.11	5.55	6.24	6.81	5.00	3.95	-
$A_P(30)$	0.00	0.00	0.73	0.00	0.00	0.00	0.10	0.00	0.12	1.00	1.32	-
H_O	0.483	0.563	0.327	0.404	0.333	0.356	0.481	0.487	0.489	0.133	0.000	0.369
H_E	0.630	0.720	0.757	0.715	0.747	0.736	0.686	0.761	0.799	0.453	0.389	0.672
F_{IS}	**0.235**	**0.220**	**0.571**	**0.438**	**0.556**	**0.520**	**0.302**	**0.363**	**0.390**	**0.713**	**1.000**	**0.440**
Null	0.11[a]	0.08[a]	0.24[a]	0.19[a]	0.23[a]	0.22[a]	0.13[a]	0.16[a]	0.17[a]	0.21[a]	0.31[a]	
CAN26												
N_A	5	5	5	4	5	4	3	4	4	0	0	6
$A_R(30)$	3.96	3.57	3.90	3.51	4.15	3.88	3.00	3.34	3.30	-	-	-
$A_P(30)$	0.15	0.33	0.03	0.00	0.09	0.02	0.00	0.00	0.00	-	-	-
H_O	0.621	0.510	0.365	0.388	0.419	0.426	0.510	0.341	0.375	0.000	0.000	0.360
H_E	0.616	0.583	0.609	0.545	0.633	0.605	0.639	0.574	0.534	0.000	0.000	0.485
F_{IS}	-0.007	0.125	**0.403**	**0.291**	**0.341**	**0.299**	0.203	**0.408**	**0.299**	-	-	**0.252**
Null	0.02	0.02	0.14[a]	0.11[a]	0.14[a]	0.11[a]	0.08	0.16[a]	0.12[a]	-	-	

continue

continued

	FLO	COR	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
CAN27												
N_A	5	4	4	4	4	4	5	3	4	3	4	9
$A_R(30)$	3.22	3.08	3.21	3.11	3.25	3.34	3.31	2.67	2.61	2.31	3.06	-
$A_P(30)$	0.14	0.00	0.38	0.01	0.01	0.02	0.29	0.00	0.31	0.00	1.22	-
H_O	0.500	0.327	0.519	0.519	0.522	0.551	0.472	0.531	0.490	0.449	0.063	0.360
H_E	0.536	0.509	0.537	0.548	0.557	0.538	0.528	0.535	0.508	0.487	0.535	0.529
F_{IS}	0.067	0.361	**0.034**	**0.055**	0.065	-0.025	0.108	0.008	0.037	0.080	**0.884**	**0.148**
Null	0.01	0.11[a]	0.04	0.05	0.00	0.00	0.06	0.00	0.00	0.02[a]	0.31	
CAN32												
N_A	5	4	4	4	4	5	4	5	5	12	10	18
$A_R(30)$	3.59	3.01	3.18	3.23	3.12	3.76	3.22	3.81	3.56	9.57	7.29	-
$A_P(30)$	0.28	0.07	0.06	0.00	0.00	0.12	0.00	0.21	0.13	4.09	1.24	-
H_O	0.333	0.163	0.259	0.352	0.239	0.383	0.327	0.400	0.347	0.723	0.638	0.379
H_E	0.336	0.225	0.310	0.363	0.305	0.337	0.347	0.349	0.367	0.849	0.700	0.408
F_{IS}	0.009	0.278	0.164	0.031	0.217	-0.140	0.059	-0.147	0.056	0.149	0.089	**0.073**
Null	0.00	0.08	0.05	0.02	0.06	0.00	0.03	0.00	0.03	0.04	0.00	
CAN33												
N_A	4	3	2	5	4	3	3	3	3	0	0	10
$A_R(30)$	2.63	2.26	1.73	2.59	2.60	2.06	1.78	1.83	1.96	-	-	-
$A_P(30)$	0.68	0.32	0.00	0.67	0.63	0.53	0.08	0.36	0.14	-	-	-
H_O	0.053	0.170	0.074	0.132	0.044	0.000	0.019	0.061	0.040	0.000	0.000	0.054
H_E	0.135	0.159	0.072	0.127	0.129	0.082	0.057	0.060	0.078	0.000	0.000	0.082
F_{IS}	**0.612**	-0.071	-0.029	-0.039	**0.657**	**1.000**	**0.665**	-0.014	0.491	-	-	**0.340**
Null	0.12[a]	0.00	0.00	0.00	0.13[a]	0.14[a]	0.09[a]	0.00	0.08	-	-	
CAN40												
N_A	12	8	11	9	10	9	9	10	8	7	6	17
$A_R(30)$	6.59	6.10	7.48	6.61	5.90	5.81	4.86	6.86	5.20	5.98	5.59	-
$A_P(30)$	0.16	0.12	0.05	0.03	0.47	0.05	0.01	0.02	0.31	1.32	1.13	-
H_O	0.466	0.449	0.463	0.385	0.261	0.457	0.327	0.447	0.367	0.148	0.194	0.360
H_E	0.612	0.542	0.654	0.616	0.521	0.605	0.424	0.646	0.554	0.663	0.748	0.599
F_{IS}	**0.241**	0.174	**0.294**	**0.378**	**0.502**	**0.248**	**0.230**	**0.311**	**0.339**	**0.780**	**0.743**	**0.368**
Null	0.08[a]	0.02	0.08[a]	0.13[a]	0.16[a]	0.09[a]	0.08[a]	0.09[a]	0.13[a]	0.31	0.32[a]	
CAN53												
N_A	4	5	5	4	7	5	5	5	4	6	4	9
$A_R(30)$	2.46	3.63	3.40	2.52	4.60	3.77	3.40	3.88	3.40	4.55	3.44	-
$A_P(30)$	0.00	0.00	0.19	0.00	0.46	0.21	0.28	0.00	0.00	0.35	0.00	-
H_O	0.155	0.306	0.222	0.185	0.370	0.245	0.302	0.306	0.245	0.633	0.292	0.296
H_E	0.176	0.308	0.224	0.203	0.409	0.279	0.313	0.326	0.308	0.563	0.266	0.599
F_{IS}	0.119	0.005	0.006	0.089	0.097	0.122	0.036	0.061	0.206	-0.124	-0.099	0.035
Null	0.03	0.00	0.00	0.03	0.04	0.04	0.00	0.03	0.06	0.00	0.00	

continue

continued

	FLO	COR	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
CAN56												
N_A	2	3	2	4	2	2	3	2	3	2	1	6
$A_R(30)$	1.26	2.17	1.63	2.29	1.33	1.68	1.58	1.31	1.83	1.37	1.00	-
$A_P(30)$	0.26	0.21	0.02	0.18	0.01	0.02	0.16	0.16	0.04	0.37	0.00	-
H_O	0.018	0.083	0.019	0.074	0.022	0.063	0.038	0.020	0.061	0.024	0.000	0.038
H_E	0.018	0.120	0.055	0.107	0.022	0.061	0.038	0.020	0.060	0.024	0.000	0.048
F_{IS}	-	0.305	0.662	0.313	-	-0.022	-0.005	-	-0.014	-	-	**0.205**
Null	0.00	0.07	0.09[a]	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
CAN60												
N_A	3	3	2	3	3	3	2	2	2	5	3	7
$A_R(30)$	2.25	2.52	2.00	2.46	2.54	2.50	1.94	1.97	1.95	3.80	2.32	-
$A_P(30)$	0.13	0.11	0.00	0.36	0.12	0.12	0.00	0.00	0.00	1.49	0.00	-
H_O	0.086	0.000	0.056	0.093	0.022	0.085	0.113	0.063	0.041	0.104	0.043	0.064
H_E	0.242	0.282	0.268	0.218	0.282	0.196	0.141	0.172	0.151	0.475	0.390	0.256
F_{IS}	**0.646**	**1.000**	**0.794**	**0.577**	**0.924**	**0.568**	0.198	**0.639**	**0.733**	**0.783**	**0.892**	**0.746**
Null	0.16[a]	0.26[a]	0.20[a]	0.12[a]	0.24[a]	0.14[a]	0.05	0.14[a]	0.15[a]	0.27[a]	0.26[a]	
Multilocus												
Mean $A_R(30)$	4.30	4.33	4.70	4.37	4.59	4.40	4.02	4.41	4.26	4.39	3.60	-
Mean $A_P(30)$	0.26	0.16	0.30	0.18	0.22	0.16	0.11	0.14	0.16	1.14	0.55	-
Mean H_O	0.364	0.349	0.340	0.344	0.335	0.358	0.359	0.351	0.348	0.217	0.157	0.320
Mean H_E	0.449	0.462	0.474	0.462	0.484	0.464	0.436	0.462	0.447	0.360	0.304	0.437
F_{IS}	**0.194**	**0.243**	**0.266**	**0.222**	**0.309**	**0.247**	**0.170**	**0.183**	**0.229**	**0.319**	**0.419**	**0.253**

N = number of samples; N_A = number of alleles; $A_R(g)$ = allelic richness (g accounts for the maximum standardized sample size i.e. twice the number of genotypes); $A_P(g)$ = private allelic richness; H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; F_{IS} = inbreeding coefficient. [a]MICRO-CHECKER detection for null alleles. Significant departure from Hardy–Weinberg equilibrium after False Discovery Rate correction is shown in bold.

Table S8. Percentage of missing data for each locus across populations (see Fig.1 for population codes).

	FLO	COR	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA
CAN9	0	0	0	0	0	0	0	0	0	100	100
CAN18	6	0	7	9	0	0	0	10	10	28	45
CAN23	0	0	0	0	0	0	0	0	0	0	0
CAN25	0	0	0	0	0	8	0	10	20	70	0
CAN26	0	0	0	9	6	0	7	16	0	100	100
CAN27	0	0	0	0	0	0	0	0	0	0	0
CAN32	0	0	0	0	0	0	0	8	0	6	0
CAN33	0	0	0	0	0	0	0	0	0	100	100
CAN40	0	0	0	0	0	6	0	0	0	46	25
CAN53	0	0	0	0	0	0	0	0	0	0	0
CAN56	0	0	0	0	0	0	0	0	0	18	35
CAN60	0	0	0	0	0	0	0	0	0	0	0

Table S9. Single-locus and multilocus F_{ST} (before and [1]after the ENA correction method) and Jost's D_{est} estimates for *Patella candei.*

Locus	F_{ST}	$^{1}F_{ST}$	D_{est}
CAN9	0.001	0.002	-
CAN18	0.061	0.054	0.436
CAN23	0.285	0.283	0.242
CAN25	0.130	0.097	0.401
CAN26	0.006	0.007	-
CAN27	0.070	0.058	0.092
CAN32	0.259	0.245	0.250
CAN33	0.000	0.018	-
CAN40	0.036	0.032	0.080
CAN53	0.019	0.017	0.009
CAN56	0.005	0.030	0.001
CAN60	0.438	0.321	0.283
All loci	0.116	0.101	0.199
95% CI	(0.047-0.192)	(0.039-0.167)	(0.194-0.204)

Table S10. Posterior probabilities of migration for individuals identified with mixed migrant ancestry. The notation [*i. j*] indexes the population source *i* and generation *j* (0 = non-migrant. 1 = 1^{ST} generation migrant. 2 = 2^{ND} generation migrant) of migrant ancestry.

Source population *#Individual*	CANARIES *#CE331*			MADEIRA *#CD318*			MADEIRA *#CD335*		
Migrant ancestry	[0.0]	[1.0]	[2.0]	[0.0]	[1.0]	[2.0]	[0.0]	[1.0]	[2.0]
	0.000	0.000	0.747	0.000	0.871	0.000	0.000	0.352	0.000
	[0.1]	[1.1]	[2.0]	[0.1]	[1.1]	[2.0]	[0.1]	[1.1]	[2.0]
	0.000	0.239	0.000	0.000	0.000	0.001	0.000	0.000	0.212
	[0.2]	[1.2]	[2.2]	[0.2]	[1.2]	[2.2]	[0.2]	[1.2]	[2.2]
	0.000	0.014	0.000	0.000	0.000	0.128	0.000	0.000	0.435

CHAPTER 5

Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): implications for conservation

ABSTRACT

The genetic erosion of populations exposed to human exploitation plays a detrimental role on a species ability to adapt to changing environmental conditions. The Macaronesia (NE Atlantic) endemic limpet *Patella aspera* (Röding 1798) has been subject to overexploitation throughout its geographic distribution. We analysed 841 limpet specimens from eleven islands across the archipelagos of Azores, Madeira and Canaries. Results from 11 nuclear microsatellite markers showed significant population structure between populations from Azores and populations from Madeira and Canaries, and absence of current or historic gene flow between these. M-ratios showed that both population clusters have experienced demographic changes over time. Heterozygote deficits were common across populations, which can be better accounted for by inbreeding than by null alleles or Wahlund effect. Such levels of inbreeding are likely a consequence of a significant reduction of reproductive units due to decades of intensive exploitation. As a sequential protandrous hermaphrodite, the size-selective harvesting of larger individuals likely fosters unbalanced sex-ratios and a consequent reproductive shortage. A recent compensatory hypothesis suggests that males are compensating the removal of larger females by undergoing sex change earlier and presumably at smaller sizes, as an adaptive response of the species under high size-biased fishing pressure. Despite such response, a dramatic reduction of N_e emerging from a large variation in the reproductive success due to overfishing and artificial genetic drift, can simply explain the inbreeding scenario observed in this Macaronesia endemic key species. This study provides valuable insights for management and conservation of *P. aspera* throughout Macaronesia.

KEYWORDS: connectivity, endemism, genetic erosion, over-exploitation, population genetics, sex-ratio

Published as:

Faria J, Pita A, Martins GM, Ribeiro PA, Hawkins SJ, Presa P, Neto AI (2017) Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): Implications for conservation. *Fisheries Research* 198: 180-188. DOI: 10.1016/j.fishres.2017.09.003

1. Introduction

The most obvious consequences of living resources uptake by humans are the reduction of the targeted population size and biomass (e.g. Christensen *et al.* 2014). Fisheries research has long provided examples of such disruptive impacts on natural systems (Hutchings and Reynolds 2004). At the population-level, fishing can alter size structure and population parameters in response to changes in species abundance (e.g. Jennings *et al.* 1999; Genner *et al.* 2010). Selective fisheries can also shift the community composition and the dynamics of an entire ecosystem, with known cascading effects on other taxa and biota (e.g. McClanahan *et al.* 1996; Myers *et al.* 2007; Casini *et al.* 2008; Smith *et al.* 2011). Moreover, the mean trophic level targeted by global fisheries has been decreasing over recent decades, shifting from large piscivorous fishes to smaller invertebrates and planktivorous fishes, leading to major changes in the structure of marine food webs (Pauly *et al.* 1998; Jackson *et al.* 2001).

Less noticeable are the genetic changes brought about by exploitation. These include the genetic subdivision of populations and the loss of genetic variation, which are thought to increase the risk of extinction via reductions in resilience and ability to recover following anthropogenic disturbances (Allendorf *et al.* 2008; Pinsky and Palumbi 2014). In fact, fisheries-induced genetic changes are known to affect a number of life-history traits that often reduce the capacity for a population to recover. For example, the most usual size-selective fishing for larger individuals can negatively affect traits such as fecundity, maturation and larval growth in many marine organisms (Walsh *et al.* 2006; Swain *et al.* 2007).

As many other fisheries, the removal of limpets has a profound impact on the ecosystem. It is known that grazing by limpets not only determines macroalgal biomass overall (Hawkins *et al.* 1992), but also modifies ecosystem stability (Coleman *et al.* 2006). In fact, current dominance of intertidal algal turfs on many islands of Macaronesia is largely attributed to the virtual absence of patellid species due to overharvesting (Martins *et al.* 2008). Moreover, algal dominance means that there is little bare rock left for the settlement of new limpet recruits.

The most economically valuable limpet species across the region is *Patella aspera* (Röding 1798), which is present in all Macaronesia archipelagos with the exception of Cape Verde, and occurs on rocky shores from the lower intertidal down to 20 m depth. *P. aspera* is a protandrous (sequential hermaphrodite) species with external fertilization, that reaches sexual maturity around 40 mm in shell length (Martins *et al.* 1987). In such species, individuals start as males, with the majority switching later in life to female. *P. aspera* is more reproductively active during winter/ early spring months with summer defined as a gonad maturation resting period (Martins *et al.* 1987; Vale 2016). Gametes are released into the water column and, upon fertilization, competent larvae can spend between to 2 to 32 days before they settle on hard substrate. These are temperature-dependent estimates that have been determined for other patellid species and may not reflect the true pelagic larvae duration of *P. aspera* (see Ribeiro 2008). *P. aspera* is assumed to have speciated from its congeneric European continental form *Patella ulyssiponensis* (Koufopanou *et al.* 1999; Weber and Hawkins 2005) probably between 8 - 4 Ma (Sá-Pinto *et al.* 2008). Lack of gene flow between insular and continental

populations, presumably after the establishment of extant ocean current patterns following the uplift of Panama isthmus, has made *P. aspera* more vulnerable to exploitation.

A key challenge of fisheries management is the correct definition of effective stocks (Hawkins *et al.* 2016). This is vital to ensure that natural resources and the fishery are managed at the appropriate spatial scale. Although the reliability of such definition may be better achieved by using multiple stock identification techniques concurrently (e.g. see holistic approach in Begg and Waldman 1999), molecular procedures have been more recently used to provide a genetic basis for aiding in stock identification (e.g. Griffiths *et al.* 2010; Dann *et al.* 2013). Moreover, molecular data can allow the understanding of the way populations in different geographic regions are connected via larval transfer or isolated from one another. In the event that connectivity exists among Macaronesia archipelagos, *P. aspera* could still maintain enough potential for local sustainability, provided that depleted stocks could be enhanced by natural gene flow from adjacent and/or more distant populations. However, experimental knowledge on such connectivity remains largely unknown so far. Here we used molecular data to identify patterns of genetic diversity and to test the null hypothesis of spatially homogeneous interbreeding and gene flow in *P. aspera* across Macaronesia, by studying the degree of genetic divergence between putative populations. We discuss possible implications of this study for fisheries management and conservation of such biologically, culturally and economically important endemic species across the region.

2. Methods

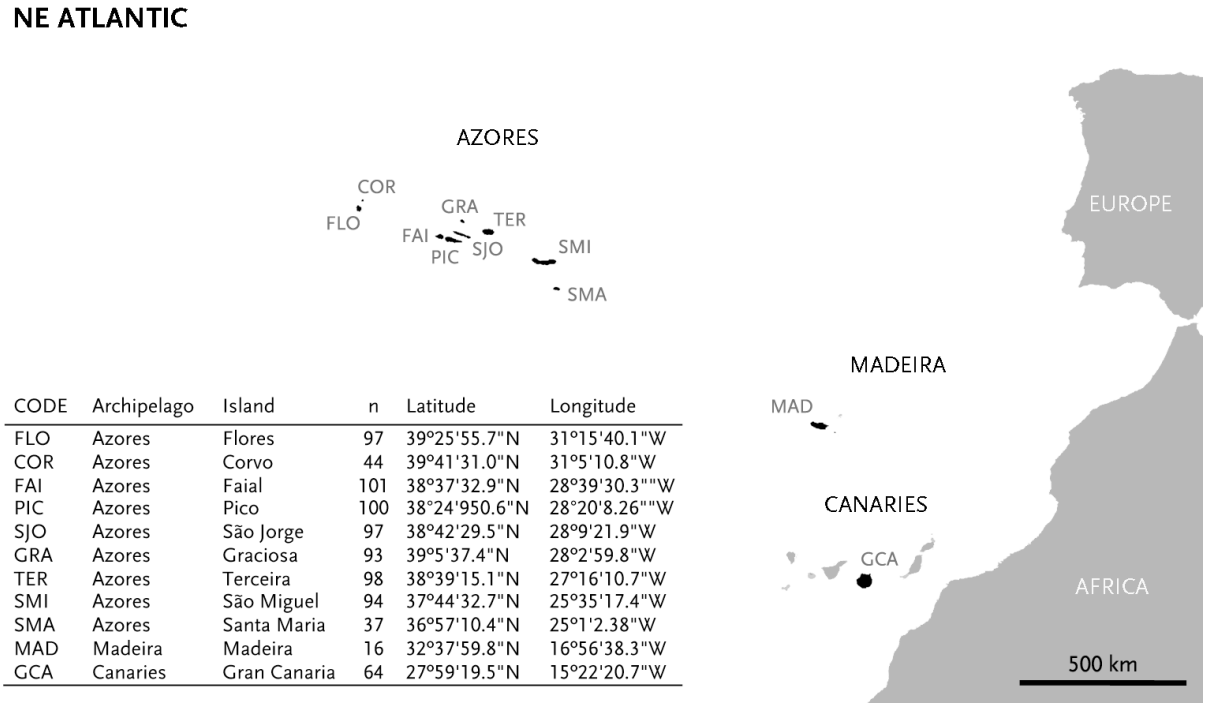
2.1. Sampling and laboratory procedures

A total of 841 individuals of *P. aspera* were collected across the Macaronesia archipelagos of Azores, Madeira and Canaries (Fig. 1). Individuals were labelled, and foot tissue samples were collected and preserved in 96% ethanol. Genomic DNA was extracted from muscle tissue using the E.Z.N.A. Mollusc DNA extraction kit following manufacturer's instructions (Omega Bio-tek). All DNA samples were quantified and checked for purity in a NanoDrop™ spectrophotometer (Thermo Scientific). Seventeen species-specific polymorphic microsatellite markers were amplified with fluorescently labelled primers following the PCR conditions described in Faria *et al.* (2015) (see Supplementary Material for methodological details).

2.2. Genetic diversity

Standard genetic diversity measures such as allele frequencies, expected (H_E) and observed (H_O) heterozygosities and inbreeding coefficients (F_{IS}) within populations for each locus and over all loci were calculated using GENALEX v.6.5 (Peakall and Smouse 2012). Deviations from Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) among all pairs of loci were tested using exact tests implemented in GENEPOP v.4.1 (Raymond and Rousset 1995). Because loci involved in significant linkage disequilibrium tests were detected, all subsequent analyses were performed on a

reduced dataset of 11 loci. Allelic richness (A_R) and private allelic richness (A_P) were calculated in HP-Rare (Kalinowski 2005) with a rarefaction sample size (in genes) of 10 due to missing data. Statistical significance for these parameters were tested with the Wilcoxon rank-sum test in R v.3.3.0 (R Core Team 2014). Results for multiple testing were adjusted by applying the false discovery rate (FDR) correction approach (Benjamini and Hochberg 1995). As large heterozygote deficits are common in marine invertebrates (Addison and Hart 2005), FREENA (Chapuis and Estoup 2007) was used to detect the presence of null alleles and quantify their frequency. Evidence for null alleles can be found in deviations to HWE, more precisely in the significant excess frequency of homozygous genotypes. Neutrality of the markers was tested using LOSITAN software (Antao *et al.* 2008) (see Supplementary Material for methodological details).



CODE	Archipelago	Island	n	Latitude	Longitude
FLO	Azores	Flores	97	39°25'55.7"N	31°15'40.1"W
COR	Azores	Corvo	44	39°41'31.0"N	31°5'10.8"W
FAI	Azores	Faial	101	38°37'32.9"N	28°39'30.3""W
PIC	Azores	Pico	100	38°24'950.6"N	28°20'8.26""W
SJO	Azores	São Jorge	97	38°42'29.5"N	28°9'21.9"W
GRA	Azores	Graciosa	93	39°5'37.4"N	28°2'59.8"W
TER	Azores	Terceira	98	38°39'15.1"N	27°16'10.7"W
SMI	Azores	São Miguel	94	37°44'32.7"N	25°35'17.4"W
SMA	Azores	Santa Maria	37	36°57'10.4"N	25°1'2.38"W
MAD	Madeira	Madeira	16	32°37'59.8"N	16°56'38.3"W
GCA	Canaries	Gran Canaria	64	27°59'19.5"N	15°22'20.7"W

Figure 1. Map of sampling locations for *Patella aspera* collected from the Macaronesia archipelagos of Azores, Madeira and Canaries (NE Atlantic).

2.3. Population structure

Genetic differentiation among population was estimated from the pairwise F_{ST} using the so-called ENA method described in Chapuis and Estoup (2007). This approach aims to correct for the positive bias induced by the presence of null alleles on F_{ST} estimation; 95% confidence intervals for the F_{ST} values were obtained using 10 000 bootstrap iterations and F_{ST} estimates obtained with and without applying the ENA algorithm were compared by means of a two tailed t-test. For comparative purposes, pairwise F_{ST} resulting from an analysis of molecular variance performed between each pair of populations (AMOVA) was also estimated in GENODIVE v.2.0b25 (Meirmans and van Tienderen 2004); statistical significance was tested by means of a permutation procedure across loci, followed by the FDR

correction for multiple testing. Genetic differentiation between populations was also determined using the D_{est} estimator (Jost 2008) implemented in the R package DEMETICS v.0.8.4 (Gerlach *et al.* 2010) and P-values were estimated by bootstrap analysis (1 000 replicates). To test for isolation by distance (Wright 1943), linearized $F_{ST\text{-}ENA}$ transformation (F_{ST} / [1- F_{ST}]) was regressed onto the natural logarithm of geographic distance (GD) (Rousset 1997) in R and tested for significance with a Mantel permutation procedure. POWSIM v.4.0 (Ryman and Palm 2006) was used to determine the power of the markers to detect significant genetic differentiation at various levels of F_{ST} (see Supplementary Material for methodological details).

Genetic population structure was assessed using the Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Pritchard *et al.* 2000) (see Supplementary Material for methodological details). The most likely number of K pools was selected using the ΔK method described in Evanno *et al.* (2005) and implemented in STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt 2012). The results of ten replicate runs for each value of K (putative number of gene pools) from 1 to 11 were averaged in CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) and summary outputs were displayed graphically in DISTRUCT v.1.1 (Rosenberg 2004). Analyses were also conducted on a reduced dataset by excluding loci with a higher frequency of null alleles (e.g. Baums *et al.* 2012). Because the high frequency of null alleles at most loci and populations might lead to overestimation of the number of K pools, the robustness of STRUCTURE results was tested using another Bayesian inference method provided by GENELAND (Guillot *et al.* 2005) and implemented in R package (see Supplementary Material for methodological details). AMOVA as implemented in ARLEQUIN v.3.5.2.2 (Excoffier and Lischer 2010) was also used to characterize the genetic structure and variance within and between clusters identified in STRUCTURE and GENELAND analyses; significance was tested after 1 000 permutations. Genetic diversity statistics were applied to each of the clusters identified with Bayesian analyses of population structure.

2.4. Unbiased estimates of inbreeding

Because homozygosity in *P. aspera* can be a consequence of harvesting-related inbreeding, the individual inbreeding model (IIM) implemented in the software INEST v.2.0 (Chybicki and Burczyk 2009) was used to simultaneously estimate null allele frequencies and inbreeding coefficients (fixation index, $F_{IS\text{-}INEST}$). This Bayesian approach allowed the calculation of unbiased estimates of inbreeding within a population after accounting for null alleles. Within INEST, two models were tested: firstly the full model which accounts for genotyping failures (*b*), inbreeding (*f*) and null alleles (*n*) and secondly the random mating model (i.e. when *f* is fixed at 0). The Deviation Information Criterion (DIC) is then used for model comparison. Support is given to an inbreeding effect when the lowest DIC is found in the *nfb* model. Also, to evaluate the effect of null alleles on F-statistics, uncorrected F_{IS} for each population was regressed onto the frequency of null alleles.

2.5. Bottleneck detection and gene flow

The heterozygosity excess test in BOTTLENECK v.1.2.02 (Cornuet and Luikart 1996) was used to test for evidence that populations had experienced recent genetic bottlenecks. Demographic declines were also assessed for the inferred clusters using the M-ratio test as implemented in M_p_val (Garza and Williamson 2001). Recent migration among clusters was estimated using the Bayesian assignment test implemented in BAYESASS v.3.0 (Wilson and Rannala 2003). All bottleneck and migration analyses were repeated after discarding loci with > 20% proportion of null alleles (see Dakin and Avise 2004) (see Supplementary Material for bottleneck and migration analyses details).

3. Results

3.1. Genetic diversity

All microsatellite loci were highly polymorphic with no significant differences in allelic richness among populations (single locus A_R ranging between 1.8 and 6.6; Table S1 and Table S2). Conversely, private allelic richness (A_P) was significantly higher in the Canaries population ($P < 0.05$) in most pairwise comparisons. Observed heterozygosity was relatively low and similar across populations with a mean value of 0.309 ± 0.165 SD (Table S1). The expected mean heterozygosity was 0.691 ± 0.154 SD and all loci deviated from HWE. The estimated frequency of null alleles ranged from 0.07 to 0.36. There was no evidence for selection at any locus (Fig. S1).

3.2. Population structure

Significant genetic differentiation (after FDR correction) was found in 34 out of 55 pairwise $F_{ST\text{-}GENODIVE}$ comparisons (Table 1). $F_{ST\text{-}GENODIVE}$ ranged from 0 to 0.114 and was not significantly different from $F_{ST\text{-}FREENA}$ corrected for null alleles (t-test, $P > 0.05$). Higher values were mainly observed between Azores and Madeira plus Canaries. D_{est} estimates were proportionally higher than F_{ST} estimates and yielded similar statistical significance values (Table 1).

Table 1. Pairwise estimates of $F_{ST\text{-}GENODIVE}$ (above diagonal) and Jost's D_{est} (below diagonal) among *Patella aspera* populations (see Fig. 1 for population codes). Significant values after FDR correction are in bold.

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA
COR	-	**0.011**	0.005	**0.010**	**0.009**	0.005	**0.010**	0.003	0.000	**0.026**	**0.086**
FLO	**0.042**	-	**0.009**	**0.005**	**0.005**	0.005	0.003	**0.008**	**0.008**	**0.060**	**0.114**
FAI	0.035	**0.034**	-	**0.006**	**0.007**	0.000	0.011	**0.006**	**0.013**	**0.035**	**0.104**
PIC	**0.050**	**0.028**	0.020	-	0.001	0.000	0.004	0.001	**0.011**	**0.042**	**0.104**
SJO	**0.053**	**0.031**	**0.035**	0.014	-	0.000	0.003	0.004	**0.010**	**0.033**	**0.092**
GRA	0.035	0.019	0.008	0.003	0.008	-	0.004	0.001	**0.008**	**0.031**	**0.097**
TER	**0.044**	**0.019**	**0.038**	0.025	0.021	0.020	-	0.003	0.006	**0.057**	**0.111**
SMI	0.022	**0.035**	0.021	0.015	**0.027**	0.013	**0.031**	-	0.002	**0.035**	**0.099**
SMA	0.011	0.043	**0.063**	**0.062**	**0.061**	**0.060**	**0.045**	0.031	-	**0.045**	**0.102**
MAD	**0.183**	**0.233**	**0.171**	**0.185**	**0.163**	**0.164**	**0.245**	**0.171**	**0.212**	-	0.020
GCA	**0.299**	**0.337**	**0.319**	**0.318**	**0.282**	**0.296**	**0.341**	**0.293**	**0.339**	0.104	-

Regression analysis showed that F-statistics might have been highly overestimated in the presence of null alleles (Fig. S2) and can therefore affect F_{ST} estimates and their significance among populations, especially on those that are putatively more closely related such as the Azorean populations. Power analysis in POWSIN indicated that the number of individuals and the number of loci used in this study provide strong support to identify weak differentiation among populations at a true F_{ST} as low as 0.0025 with a statistical power of 100% for both Fisher's exact test and the chi-square test.

There was a strong signature of isolation by distance among archipelagos (Fig. 2, $r^2 = 0.541$; $P < 0.001$), but genetic differentiation within the Azores did not seem to relate to geographic distance (Fig. 2, $r^2 = 0.069$; $P > 0.12$). STRUCTURE analyses performed under mixed ancestry and sampling locations as prior information, and the ΔK metric of Evanno *et al.* (2005) revealed that K = 2 was the most likely number of gene pools/ clusters for *P. aspera* in Macaronesia (Fig. 3 and Fig. S3, respectively). Two distinct clusters were identified: one involving all Azorean populations and another grouping Madeira and Canaries. Population structure became less evident when analytical parameters and settings were changed in STRUCTURE, especially when prior information for population origin was not implemented (see Fig. S4). The spatial model in GENELAND, bearing a correction for null alleles, estimated the number of clusters for *P. aspera* in Macaronesia as two, in agreement with initial STRUCTURE results (Fig. 3): a northern group encompassing all Azorean populations and a southern group including Madeira and Canaries populations. A significant genetic differentiation among clusters was also detected through AMOVA analyses; most of the total variance was due to variation within populations (Table S3).

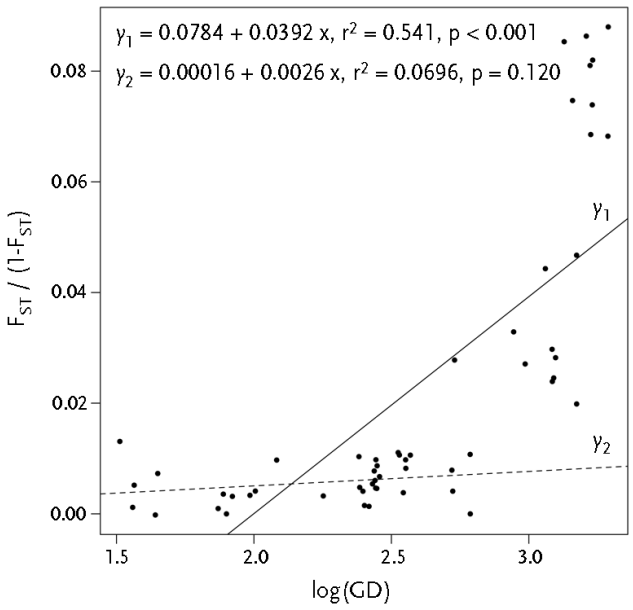


Figure 2. Regression between genetic distances $F_{ST}/(1 - F_{ST})$ and geographical distances (GD) among and within archipelagos (y_1 and y_2, respectively).

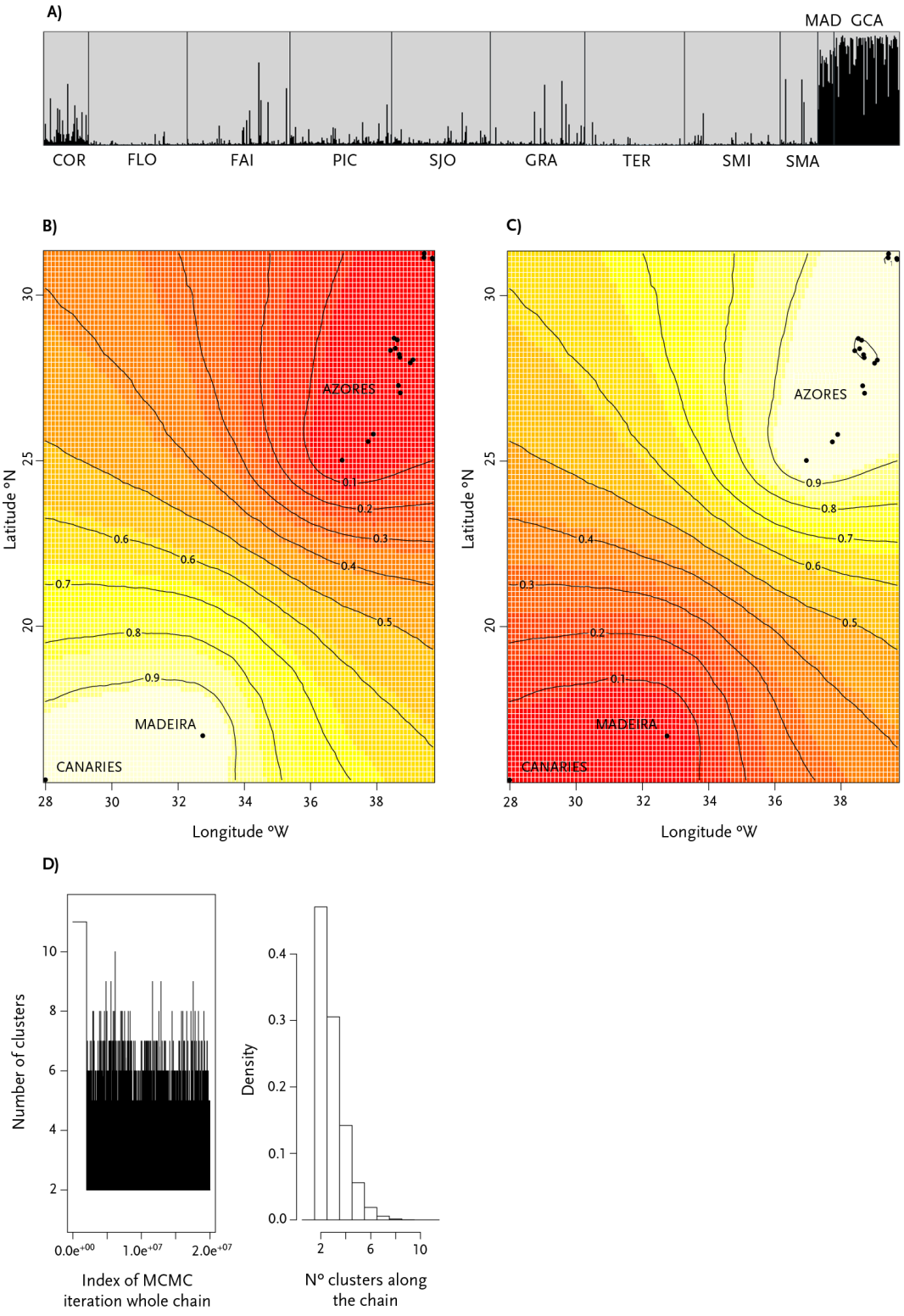


Figure 3. Bayesian analysis results for *Patella aspera* using 11 loci following: A) STRUCTURE clustering analysis considering admixture and prior information for population origin; bar graph show the average probability of membership (y-axis) of individuals (n = 841, x-axis) in K = 2 clusters (see Fig. 1 for population codes); B, C) GENELAND spatial analysis showing the assignment of individuals to clusters for K = 1 and K = 2, respectively; darker and lighter shading are proportional to posterior probabilities of membership in each cluster, with lighter (yellow) areas showing the highest probabilities of clusters; D) posterior density distribution of the number of clusters (clear mode at K = 2).

The genetic diversity of each cluster was similar to those obtained for population/location level analyses (Table 2). Mean observed and expected heterozygosity in the first cluster (all samples from Azores) were 0.315 ± 0.160 SD and 0.701 ± 0.150 SD, respectively. For the second cluster (Madeira and Canaries samples), the observed mean heterozygosity was 0.285 ± 0.173 SD and the expected mean heterozygosity was 0.697 ± 0.188 SD.

Table 2. Genetic variation observed at eleven microsatellite loci within two clusters of *P. aspera* identified by STRUCTURE and GENELAND analyses.

Cluster/ Locus	ASP2	ASP3	ASP7	ASP17	ASP21	ASP27	ASP29	ASP33	ASP36	ASP38	ASP39	ALL
AZORES												
N_A	15	10	11	14	17	19	39	20	22	20	13	
$A_R(12)$	5.0	4.1	2.2	5.1	5.2	4.8	6.5	5.9	5.9	4.5	2.8	4.7
$A_P(12)$	1.4	1.2	1.2	2.1	2.1	1.2	3.8	3.0	3.0	2.0	1.3	2.0
H_O	0.611	0.200	0.085	0.292	0.559	0.323	0.253	0.428	0.304	0.252	0.162	0.315
H_E	0.795	0.589	0.509	0.774	0.784	0.774	0.836	0.773	0.811	0.701	0.363	0.701
F_{IS}	0.232	0.660	0.834	0.620	0.289	0.581	0.702	0.449	0.626	0.638	0.555	0.399[a]
Null	0.10	0.25	0.28	0.27	0.13	0.25	0.28	0.20	0.28	0.27	0.18	
MADEIRA + CANARIES												
N_A	12	9	3	9	12	11	21	19	12	11	3	
$A_R(12)$	6.7	5.4	7.0	4.3	4.2	4.4	6.7	7.1	5.2	5.1	1.9	5.3
$A_P(12)$	3.2	2.4	5.9	1.4	1.1	0.8	4.0	4.2	2.2	2.6	0.4	2.6
H_O	0.123	0.098	0.000	0.230	0.437	0.197	0.310	0.557	0.409	0.349	0.091	0.285
H_E	0.818	0.802	0.370	0.653	0.695	0.782	0.784	0.876	0.808	0.745	0.292	0.697
F_{IS}	0.876	0.869	0.833	0.676	0.287	0.791	0.627	0.359	0.475	0.514	0.792	0.104[a]
Null	0.40	0.39	0.37	0.27	0.12	0.35	0.28	0.15	0.20	0.22	0.17	

N_A = number of alleles; $A_R(g)$ = allelic richness (g accounts for the maximum standardized sample size i.e. twice the number of genotypes); $A_P(g)$ = private allelic richness; H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; F_{IS} = uncorrected inbreeding coefficient; [a]"null free" inbreeding coefficient $F_{IS\text{-}INEST}$ for each cluster. Null = frequency of null alleles.

3.3. Inbreeding

The multilocus "null free" average inbreeding coefficient (F_{IS}) as calculated with INEST varied between 0.113 and 0.497 (Table S1). Model comparison using INEST suggests that the heterozygote deficit observed in the dataset is better accounted for by inbreeding than by null alleles. In all cases, the *nfb* model had the lowest DIC (Table S4). Similarly, model comparison with INEST on the two clusters identified through Bayesian analyses also indicated that heterozygosities deficits are more related to inbreeding rather then to null alleles presence: the "null free" inbreeding coefficient $F_{IS\text{-}INEST}$ for each cluster was 0.399 (95% confidence interval: 0.368 - 0.431) and 0.104 (95% confidence interval: 0.043 - 0.272), respectively (Table 2).

3.4. Bottleneck detection and gene flow

The heterozygosity excess test implemented in BOTTLENECK did not find evidence for a recent demographic bottleneck in any of the clusters assumed (Table S5; $P > 0.05$). Bottleneck detection using the M-ratio method did, however, show that clusters have experienced demographic changes in time. Although the performance of the method is dependent on initial assumptions and settings, the more conservative approach recommended by Garza and Williamson (2001) indicated a recent reduction in population size, within the last few hundred generations, for both Azores and Madeira plus Canaries clusters (Fig. S5). In such case M-ratios for individual clusters fell under the lower 5% of the distribution of simulated M-values. Setting the average size of non one-step mutations to 2.8, which is the mean value for this parameter in the literature (see Garza and Williamson 2001), increased the detection of bottlenecks at higher values of the pre-bottleneck Θ (Fig. S5). Estimates of contemporary migration rates calculated with BAYESASS showed no signs of gene flow between clusters (Table S6). Results of bottleneck and migration analyses did not differ when using a reduced loci dataset, i.e. only loci with < 20% proportion of null alleles.

4. Discussion

Even though a high dispersal potential is expected in *P. aspera* as drawn from the pelagic larval duration of its congeneric European continental form *P. ulyssiponensis,* which is estimated to range between 14.5 - 27 days (average age of planktonic life *in vitro* after metamorphic competence; see Ribeiro 2008), unfavourable oceanographic conditions, large larvae mortality and the relatively large geographical distance among populations may have prompted the isolation between Azores and those on the remainder of the Macaronesia archipelagos. In fact, larval transport may be strongly affected by local eddies, current reversals and other unknown oceanographic physical patterns (Sponaugle *et al.* 2002; Palumbi 2004). Moreover, marine larvae often face a huge mortality over relatively short distances from their spawning locations (e.g. Cowen *et al.* 2000). Larvae biological traits (e.g. growth, swimming and orientation capacities, reproductive and recruitment strategies) and species interactions (e.g. predation, food availability) may also determine a limited connectivity within a given species (Cowen and Sponaugle 2009). In fact, genetic breaks in marine invertebrates with high putative dispersal capacity are not uncommon in the literature (see Galarza *et al.* 2009; Serrano *et al.* 2014; Ouagajjou and Presa 2015; Guiñez *et al.* 2016). The relatively fragile nature of patellid larvae (when compared, for instance, with crustacean larvae), the realized larval pelagic duration which is presumably shorter in the field (e.g. Olson and McPherson 1987) and the predominately oligotrophic character of NE Atlantic waters surrounding the Macaronesia archipelagos (Marañon *et al.* 2000; Silva *et al.* 2013), may act to favour local retention and limit the dispersal capacity of *P. aspera* over large distances. Thus, genetic differentiation in *P. aspera* probably arose from the historical and contemporary complex interplay of particular biological and physical processes that favoured isolation over population homogeneity. Nevertheless, the shortest distance between Madeira and Canaries and the presence of the Selvagens islands in between both archipelagos, are likely to facilitate larvae dispersal and population connectivity of *P. aspera* among those archipelagos.

High inbreeding coefficients were detected in all populations. Such large deviations from HWE and excess of homozygotes are often strongly linked to the occurrence of null alleles across loci, with most population genetic studies avoiding their use in analyses. However, deviations from HWE can also be biological meaningful, especially when such deviations occur at multiple loci, and may be explained by several nonexclusive factors such as inbreeding, selection against heterozygotes and the Wahlund effect (Dakin and Avise 2004; Allendorf and Luikart 2007). The individual inbreeding model implemented in INEST showed that, although null alleles have an undoubted role on heterozygote deficit and interpopulation differentiation, inbreeding appears as the most likely process underpinning the observed positive F_{IS} values. Processes that lead to inbreeding in natural populations (e.g. non-random mating) are often considered to be more effective and associated with small populations and would hardly affect marine populations with expected high census population sizes. However, stochastic factors acting upon the recruitment success and especially the anthropogenic influence on the population dynamics and demography can lead to an unbalanced genetic composition in widespread marine populations (see O'Leary *et al.* 2013; Aglieri *et al.* 2014). Such processes can result in unexpected levels of relatedness among individuals and therefore inbreeding rates higher than expected. Exploitation of *P. aspera* across Macaronesia not only has reduced its abundance, but also the mean size of individuals (see section below). Since *P. aspera* is a protandric hermaphrodite, therefore undergoing sex change from male into female as they grow, harvesting is strongly biased towards females. Such reduction in the number of females is believed to trigger non-random mating and the production of individuals which relatedness is higher than expected by chance. In such over-harvested populations, the census size (N) can thus greatly differ from the effective population size (N_e), where the number of individuals that actively contributes for the next generation being rather small. In fact, a reduction in N_e would counter the mechanisms that promote random mating in *P. aspera*, such as high fecundity, external fertilization with broadcast spawning, and putative extensive larval dispersal. Moreover, the fact that harvesting is size selective towards the larger individuals may impose a strong selection among heritable characteristics within exploited populations (see Conover and Munch 2002). Noteworthy, recent research has revealed that the sex-ratio of *P. aspera* remained unchanged regardless the increasing fishing pressure, suggesting that males are compensating for the removal of larger females by undergoing sex change at smaller and presumably earlier sizes (Martins *et al.* 2017), a pattern that has also been observed in other patellid limpets (Borges *et al.* 2016). Although this mechanism can buffer populations against changes in sex-ratio of the species, the fact that females became smaller than their historical size, would affect the amount of gametes released into the water, with obvious negative consequences for the reproductive output of *P. aspera*. Moreover, it is still undetermined how this compensatory mechanism is to affect the gametic fitness of such small-size females or its impact on larval viability and dispersal capacity. Under these circumstances, inbreeding can emerge from a large variation in individual reproductive success influenced by particular environmental, reproductive and behaviour factors, with only a few individual limpets reproducing and actively contributing to the genetic pool of the next generation (see Hedgecock 1994). Although inbreeding is suggested as the main driver for the observed excess of homozygotes in *P. aspera* populations, other alternative mechanisms such as extensive within-

population genetic structure (i.e. the Wahlund effect), higher rates of molecular evolution and randomly induced differences in allele frequencies between sperm and eggs have also been proposed to explain deviations from HWE in free-spawning marine invertebrates (see Addison and Hart 2005). In fact, the occurrence of a Wahlund effect cannot be entirely discarded, as unrecognized temporal breeding subunits may occur inside sampled populations, resulting in large variations from HWE. Moreover, seasonal or inter-annual changes in currents might bring together larvae from locally differentiated sources, providing the baseline for genetic patchiness and spatial substructuring of within-population demes. Yet, a Wahlund effect is less likely in our study, since each local geographic sample was tested separately and Bayesian clustering analyses did not detect any within-sample structuring, or within archipelago for that matter (i.e. Azores). Preliminary analyses for within-island genetic variation (two sampled locations within each island) also showed the absence of any population structuring at such smaller spatial scale. Furthermore, the impact of the Wahlund effect is usually more substantial when cryptic populations, recipient and donor, exhibit large differences in gene diversity (Zhivotovsky 2015); in this case, independently of distance, all *P. aspera* populations are relatively genetic impoverished with similar diversity, have individuals with alike morphological traits and there are no apparent differentiated niches where putative cohorts could genetically thrive distinctively. For instance, Côrte-Real *et al.* (1992) found no genetic differences between intertidal and subtidal specimens of *P. aspera* in the Azores. More importantly, although homozygote excess was shown to be surprisingly common in free-spawning species (see Addison and Hart 2005), deviations to HWE should be, above all, interpreted under the life-cycle particularities and circumstances of a given species. As so, given the acceptable sample size and number of microsatellite markers used in this study, the genome-wide effect detected (i.e. significant heterozygote deficits across all loci screened in all populations studied), the specificities of *P. aspera* reproductive/ breeding cycle and the unbalanced nature of harvesting, inbreeding seems the most plausible mechanism for the observed excess of homozygotes in *P. aspera* populations from Macaronesia. The retention of planktonic larvae near parent habitats, which is not uncommon even in species with very high dispersal potential (e.g. Swearer *et al.* 1999), may further support the idea that there are no high levels of immigration from other sites that otherwise would reduce the chances of inbreeding. A deeper insight into the spatial genetic structure of *P. aspera* populations would be possible by taking the so-called 'seascape genetics' approach, and use oceanographic modelling (e.g. Galindo *et al.* 2006) as well as many other interacting forces and traits to understand the processes involved in marine connectivity of the species (see review by Selkoe *et al.* 2016)

Studies that include the effect of overexploitation or a reduction in the effective population size on the genetic diversity and inbreeding phenomena of marine invertebrates are fairly scarce (e.g. Gallardo *et al.* 1998; Constantini *et al.* 2007), and examples are often provided for other organisms such as fishes (e.g. Hutchinson *et al.* 2003; Hoarau *et al.* 2005; O'Leary *et al.* 2013). The generalized idea is that inbreeding, although occurring naturally in many populations, is intensified by overexploitation reducing the capability for adaptation and increasing the extinction risk (Keller and Waller 2002; Frankham 2005). Under these circumstances, exploited populations will tend to suffer to a greater extent from demographic and genetic stochasticity and face higher chances of extinction due to

inbreeding or loss of genetic diversity by drift (Frankham *et al.* 2014). Population subdivision can also foster inbreeding by decreasing migrants among subpopulations and prompting chances for mating among relatives (Bierne *et al.* 1998; Andrade and Solferini 2007). In such case, and even assuming random mating, inbreeding could occur because population sizes are very limited and the consequences of genetic drift become apparent (Keller and Waller 2002). In fact, population differentiation in *P. aspera* given by pairwise distances estimates (F_{ST} and D_{est}) indicated some degree of subdivision among islands (see Table 2). These estimates, however, may be unreliable, and interpretation should be cautious due to the known impact of null alleles in overestimating population differentiation. With much greater support, our findings suggested that i) Azores samples comprise an isolated population and ii) Madeira and Canaries populations cluster together in a single group. Moreover, insular populations of *P. aspera* are thought to have been isolated from continental forms for the last 4 - 8 Ma (Sá-Pinto *et al.* 2005; 2008). These same studies, using mtDNA analyses, revealed genetic differentiation between Azores and the remaining archipelagos. Given the high level of isolation and the similarity in environmental conditions among archipelagos, genetic diversity is not expected to be high. Whereas genetic diversity, under these circumstances, can be lost purely due to genetic drift, the prolonged and long-time harvesting of limpets across archipelagos is likely to have negatively impacted genetic diversity and favoured inbreeding due to a reduction in the effective population size in *P. aspera*. Unfortunately, there are no current estimates of *P. aspera* effective population sizes throughout the region. Yet, considering the historical and more recent Catch Per Unit Effort (CPUE) as a reliable proxy of population census sizes in *P. aspera*, i.e. in the early 1990's there were still places in Azores where CPUE was around 12 kg / half hour but this has fallen to 5 g / half hour in recent years (OSPAR-Commission (2010), it is clear that a dramatic reduction in the abundance of animals has taken place, which is likely to have negatively affected the present-day N_e in *P. aspera* across the region.

4.1. Implications for the management and conservation

Harvesting of *P. aspera*, which is currently listed in the OSPAR list of threatened and/or declining species and habitats (OSPAR-Commission 2010), is under specific legislation throughout the Macaronesia region. At each archipelago, seasonal fishing closures and minimum catch sizes have been imposed: Azores (October - April, 50 mm); Madeira (December-March, 40 mm); and Canaries (December - April, 45 mm). Moreover, harvesting is forbidden inside specific marine protected areas and/or limpet protection zones in each archipelago. Catch limits (kg) per day for commercial (with permit only) and recreational use have also been established. Seasonal closures have broadly been defined in agreement with reproductive periods occurring during colder months (see Vale 2016). Despite this, non-compliance with current legislation is the norm among limpet harvesters (see Diogo *et al.* 2016). Empirical evidence suggests that *P. aspera* stocks are pretty much depleted and overexploited (Martins *et al.* 2008; 2011) and the lack of enforcement of regulations is seen as the most likely reason for the failure to protect limpet populations. To raise awareness, regional authorities should promote environmental education actions directed to fishermen and general public, for the

importance of the sustainable use of limpets as a resource as well as a key ecological asset. Limpet protection zones, which have been shown to be largely ineffective (see Martins *et al.* 2011; Diogo *et al.* 2016), would likely work if permanently monitored and surveyed by local authorities. Management approaches should also target intermediate-sized individuals by establishing an upper size limit on limpets (Conover and Munch 2002; Allendorf *et al.* 2008). In the short term, this would increase the number of larger females potentially generating higher-quality offspring and contributing to an increasing effective population size in *P. aspera* (see Birkeland and Dayton 2005) and thus helping reducing inbreeding rates. From a stock perspective, and considering the population differentiation found among the Azorean and other archipelagos' populations, both Madeira and Canaries authorities should join efforts in establishing a common management strategy for the protection and harvesting of *P. aspera* in those archipelagos. Moreover, the high levels of isolation and exploitation of the Azorean populations suggests that authorities must act immediately to avoid the genetic and demographic depletion of *P. aspera* stocks in the Azores. Scientifically sound data on this limpet reproductive cycle, its recruitment rate and its demographic status would help inform conservation actions and formal stock definition. Although genetic approaches are only a small piece in the intricate puzzle of defining effective fishery stocks, this study suggests that limpet conservation and fisheries management across Macaronesia should, at the very least, consider two independent stocks: one in Azores and another one in Madeira - Canaries. Management and conservation initiatives need to be revisited in the light of such results. Above all, the most direct way to reduce the effects of over-exploitation on *P. aspera* across the Macaronesian region is to drastically reduce the intensity of harvest of its populations.

Funding

This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE − Operational Competitiveness Programme and national funds through FCT − Foundation for Science and Technology, under the projects PTDC/BIA- BIC/115837/2009 and PEst-C/MAR/LA0015/2013, by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT − Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020 and by cE3c funding (Ref: UID/BIA/00329/2013). JF was funded by a PhD grant M3.1.2/F/021/2011 by the Regional Government of the Azores. AP was supported by a post-doctoral grant from Xunta de Galicia, Spain (P.P. 0000 421S 140.08. GMM was sup- ported by post-doctoral grants awarded by FCT, Portugal (SFRH/BDP/ 63040/2009). PAR was funded by the Portuguese Foundation for Science and Technology, through a postdoctoral grant ref. SFRH/BPD/ 69232/2010 funded through QREN and COMPETE, and the strategic project UID/MAR/04292/2013 granted to MARE.

Acknowledgments

We sincerely thank two anonymous reviewers for their useful comments that improved significantly this manuscript. We thank Manuel Rivas for helping with DNA extractions. Field and sampling

assistance was provided by Joana Pombo in Santa Maria (Azores), José Azevedo in Pico (Azores), André Amaral in Terceira (Azores), Pedro Raposeiro in Flores and Corvo (Azores), and Fernando Tuya and Manuel Rivas in Canarias. We thank Leopoldo Moro (Government of Canarias) for providing information on limpet harvesting regulations in Canaries.

References

Addison JA, Hart MW (2005) Spawning, copulation and inbreeding coefficients in marine invertebrates. *Biology Letters* 1: 450-453.

Aglieri G, Papetti C, Zane L, Milisenda G, Boero F, Piraino S (2014) First evidence of inbreeding, relatedness and chaotic genetic patchiness in the holoplanktonic jellyfish *Pelagia noctiluca* (Scyphozoa, Cnidaria). *Plos One* 6: e99647.

Allendorf FW, England PR, Luikart G, Ritchie PA Ryman N (2008) Genetic effects of harvest on wild animal populations. *Trends in Ecology and Evolution* 23: 327-337.

Allendorf FW, Luikart G (2007) *Conservation and the genetics of populations*. Blackwell Publishing, London.

Andrade SCS, Solferini VN (2007) Fine-scale genetic structure overrides macro-scale structure in a marine snail: nonrandom recruitment, demographic events or selection?. *Biological Journal of the Linnean Society* 91: 23-36.

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics* 9: 323.

Baums IB, Boulay JN, Polato NR, Hellberg ME (2012) No gene flow across the Eastern Pacific Barrier in the reef-building coral *Porites lobata*. *Molecular Ecology* 21: 5418-5433.

Begg GA, Waldman JR (1999) An holistic approach to fish stock identification. *Fisheries Research* 43: 35-44.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A practical and powerful approach to multiple testing. *The Journal of the Royal Statistical Society B* 57: 289-300.

Bierne N, Launey S, Naciri-Graven Y, Bonhomme F (1998) Early Effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. *Genetics* 148: 1893-1906.

Birkeland C, Dayton PK (2005) The importance in fishery management of leaving the big ones. *Trends in Ecology and Evolution* 20: 356-358.

Borges CDG, Hawkins SJ, Crowe TP, Doncaster CP (2016) The influence of simulated exploitation on *Patella vulgata* populations: protandric sex change is size-dependent. *Ecology and Evolution* 6(2): 514-531.

Casini M, Lövgren J, Hjelm J, Cardinale M, Molinero JC, Kornilovs G (2008) Multi-level trophic cascades in a heavily exploited open marine ecosystem. *Proceedings of the Royal Society of London B: Biological Sciences* 275: 1793-1801.

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621-631.

Christensen V, Coll M, Piroddi C, Steenbeek J, Buszowski J, Pauly D (2014) A century of fish biomass decline in the ocean. *Marine Ecology Progress Series* 512: 155-166.

Chybicki IJ, Burczyk J (2009) Simultaneous estimation of null alleles and inbreeding coefficients. *Journal of Heredity* 100: 106-113.

Coleman RA, Underwood AJ, Benedetti-Cecchi, Aberg P, Arenas F, Arrontes J, Castro J, Hartnoll RG, Jenkins SR, Paula J, Santina PD, Hawkins SJ (2006) A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia* 147: 556-564.

Conover DO, Munch SB (2002) Sustaining fisheries yields over evolutionary time scales. *Science* 297: 94-96.

Constantini F, Fauvelot C, Abbiati M (2007) Fine-scale genetic structuring in *Corallium rubrum*: evidence of inbreeding and limited effective larval dispersal. *Marine Ecology Progress Series* 340: 109-119.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144: 2001-2014.

Côrte-Real HSM, Hawkins SJ, Thorpe JP (1992) Genetic confirmation that intertidal and subtidal morphs of *Patella ulyssiponensis aspera* Röding (Mollusca: Gastropoda: Patellidae) are conspecific. *Arquipélago* 10: 55-66.

Cowen RK, Lwiza KMM, Sponaugle S, Paris CB, Olson DB (2000) Connectivity of marine populations: open or closed?. *Science* 287: 857-859.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review of Marine Science* 1: 443-466.

Dakin EE, Avise JC (2004) Microsatellite null alleles in parentage analysis. *Heredity* 93(5): 504-509.

Dann TH, Habicht C, Baker TT, Seeb JE (2013) Exploiting genetic diversity to balance conservation and harvest of migratory salmon. *Canadian Journal of Fisheries and Aquatic Sciences* 70: 785-793.

Diogo H, Pereira JG, Schmiing M (2016) Catch me if you can: Non-compliance of limpet protection in the Azores. *Marine Policy* 63: 92-99.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetic Resources* 4: 359-361.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611-2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10: 564-567.

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46(1): 96-97.

Frankham R (2005) Genetics and extinction. *Biological Conservation* 126: 131-33.

Frankham R, Bradshaw CJA, Brook BW (2014) Genetics in conservation management: revised recommendations for the 50/500 rules, red list criteria and population viability analyses. *Biological Conservation* 170: 56-63.

Galarza JA, Carreras-Carbonell JC, Macpherson E, Pascual M, Roques S, Turner GF, Rico C (2009) The influence of oceanographic fronts and early-life-history traits on connectivity among littoral fish species. *Proceedings of the National Academy of Sciences of USA* 106: 1473-1478.

Galindo HM, Olson DB, Palumbi SR (2006) Seascape genetics: a coupled oceanographic-genetic model predicts population structure of Caribbean corals. Current Biology 16: 1622-1626.

Gallardo MH, Peñaloza L, Clasing E (1998) Gene flow and allozymic population structure in the clam *Venus antiqua* (King of Broderip), (Bivalvia, Veneriidae) from Southern Chile. *Journal of Experimental Marine Biology and Ecology* 230: 193-205.

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* 10: 305-318.

Genner MJ, Sims DW, Southward AJ, Budd GC, Masterson P, McHugh M, Rendle P, Southall EJ, Wearmouth VJ, Hawkins SJ (2010) Body size-dependent responses of a marine fish assemblage to climate change and fishing over a century-long scale. *Global Change Biology* 16: 517-527.

Gerlach G, Jueterbock A, Kraemer P, Deppermann J, Harmand P (2010) Calculations of population differentiation based on $G_{(ST)}$ and D: forget $G_{(ST)}$ but not all of statistics! *Molecular Ecology* 19: 3845-3852.

Griffiths AM, Machado-Schiaffino G, Dillane E, Coughlan J, Horreo JL, Bowkett AE, Minting P, Toms S, Roche W, Gargan P, McGinnity P, Cross T, Bright D, Garcia-Vazquez E, Stevens JR (2010) Genetic stock identification of Atlantic salmon (*Salmo salar*) populations in the southern part of the European range. *BMC Genetics* 11:31.

Guillot G, Mortier F, Estoup A (2005) GENELAND: a program for landscape genetics. *Molecular Ecology Resources* 5: 712-715.

Guiñez R, Pita A, Pérez M, Briones C, Navarrete SA, Toro J, Presa P (2016) Present-day connectivity of historical stocks of the ecosystem engineer *Perumytilus purpuratus* along 4500 km of the Chilean Coast. *Fisheries Research* 179: 322-332.

Hawkins SJ, Hartnoll RG, Kain JM, Norton TA (1992) Plant-animal interactions on hard substrata m the North-West Atlantic. In *Plant-Animal Interactions in the Marine Benthos*. John DM, Hawkins SJ, Price JH (Eds). The Systematics Association, Special Vol. 46. Clarendon Press, Oxford. pp. l-32.

Hawkins SJ, Bohn K, Sima DW, Ribeiro P, Faria J, Presa P, Pita A, Martins GM, Neto AI, Burrows MT, Genner MJ (2016) Fisheries stocks from an ecological perspective: Disentangling ecological connectivity from genetic interchange. *Fisheries Research* 179: 333-341.

Hedgecock D (1994) Does variance in reproductive success limit effective population sizes of marine organisms?. In G*enetics and evolution of aquatic organisms.* Beaumont AR (Ed). Chapman and Hall. pp 122-134.

Hoarau G, Boon E, Jongma DN, Ferber S, Palsson J, Van der Veer HW, Rijnsdorp D, Stam WT, Olsen JL (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (*Pleuronectes platessa* L.). *Proceedings of the Royal Society of London B: Biological Sciences* 272: 497-503.

Hutchings JA, Reynolds JD (2004) Marine fish population collapses: Consequences for recovery and extinction risk. *BioScience* 54(4): 297-309.

Hutchinson WF, van Oosterhout C, Rogers SI, Carvalho GR (2003) Temporal analysis of archived samples indicates marked genetic changes in declining North Sea cod (*Gadus morhua*). *Proceedings of the Royal Society of London B: Biological Sciences* 270: 2125-2132.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, Bradbury RH, Cooke R, Erlandson J, Estes JA, Hughes TP, Kidwell S, Lange CB, Lenihan HS, Pandolfi JM, Peterson CH, Steneck RS, Tegner MJ, Warner RR (2001) Historical overfishing and the recent collapse of coastal ecosystems. *Science* 293: 629-638.

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23: 1801-1806.

Jennings S, Greenstreet SPR, Reynolds JD (1999) Structural change in an exploited fish community a consequence of differential fishing effects on species with contrasting life histories. *Journal of Animal Ecology* 68: 617-627.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. *Molecular Ecology* 17: 4015-4026.

Kalinowski ST (2005) HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. *Molecular Ecology Notes* 5: 187-189.

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecology and Evolution* 17: 230-241.

Koufopanou V, Reid DG, Ridgway SA, Thomas RH (1999) A molecular phylogeny of the patellid limpets (Gastropoda: Patellidae) and its implications for the origins of their antitropical distribution. *Molecular Phylogenetics and Evolution* 11: 138-156.

Marañon E, Holligan PM, Varela M, Mouriño B, Bale AJ (2000) Basin-scale variability of phytoplankton biomass, production and growth in the Atlantic Ocean. *Deep-Sea Research I* 47: 825-857.

Martins HR, Santos RS, Hawkins SJ (1987) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Medeiros AR, Thompson RC (2011) Illegal harvesting affects the success of fishing closure areas. *Journal of the Marine Biological Association UK* 91: 929-937.

Martins GM, Borges CDG, Vale M, Ferraz RR, Martins HR, Santos RS, Hawkins SJ (2017) Exploitation promotes earlier sex change in a protandrous patellid limpet, *Patella aspera* Röding, 1798. *Ecology and Evolution* 7(10): 3616-3622.

McClanahan TR, Kamukuru AT, Muthiga NA, Gilagabher M, Obura D (1996) Effect of sea urchin reductions on algae, coral and fish populations. *Conservation Biology* 10: 136-154.

Meirmans PG, van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792-794.

Myers RA, Baum JK, Shepherd TD, Powers SP, Peterson CH (2007) Cascading effects of the loss of apex predatory sharks from a coastal ocean. *Science* 315: 1846-1850.

O'Leary SJ, Hice LA, Feldheim KA, Frisk MG, McElroy AE, Fast MD, Chapman DD (2013) Severe inbreeding and small effective number of breeders in a formerly abundant marine fish. *Plos One* 8: e66126.

Olson RR, McPherson R (1987) Potential vs. realized larval dispersal: fish predation on larvae of the ascidian *Lissoclinum patella* (Gottschaldt). *Journal of Experimental Marine Biology and Ecology* 110: 245-256.

OSPAR-Commission (2010) Background document for Azorean limpet *Patella aspera*.

Ouagajjou Y, Presa P (2015) The connectivity of *Mytilus galloprovincialis* in northern Morocco: A gene flow crossroads between continents. *Estuarine, Coastal and Shelf Science* 152: 1-10.

Palumbi SR (2004) Marine reserves and ocean neighborhoods: The spatial scale of marine populations and their management. *Annual Review of Environment and Resources* 29: 31-68.

Pauly D, Christensen V, Dalsgaard J, Froese R, Torres F (1998) Fishing down marine food webs. *Science* 279: 860-863.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28: 2537-2539.

Pinsky ML, Palumbi SR (2014) Meta-analysis reveals lower genetic diversity in overfished populations. *Molecular Ecology* 23: 29-39.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

R Core Team (2014) R: *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/.

Raymond M, Rousset F (1995) GENEPOP (version 1.2) - population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Rosenberg NA (2004) Distruct: a program for the graphical display of population structure. *Molecular Ecology Resources* 4: 137-138.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* 145: 1219-1228.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes* 6: 600-602.

Sá-Pinto A, Branco MS, Harris DJ, Alexandrino P (2005) Phylogeny and phylogeography of the genus *Patella* based on mitochondrial DNA sequence data. *Journal of Experimental Marine Biology and Ecology* 325: 95-110.

Sá-Pinto A, Branco A, Sayanda D, Alexandrino P (2008) Patterns of colonization, evolution and gene flow in species of the genus *Patella* in the Macaronesian Islands. *Molecular Ecology* 17: 519-532.

Selkoe KA, D'Aloia CC, Crandall ED, Iacchei M, Liggins L, Puritz JB, von der Heyden S, Toonen RJ (2016) A decade of seascape genetics: contributions to basic and applied marine connectivity. *Marine Ecology Progress Series* 554: 1-19

Serrano X, Baums IB, O'Reilly K, Smith TB, Jones RJ, Shearer TL, Nunes FLD, Baker AC (2014) Geographic differences in vertical connectivity in the Caribbean coral Montastraea cavernosa despite high levels of horizontal connectivity at shallow depths. *Molecular Ecology* 23: 4226-4240.

Silva A, Brotas V, Valente A, Sá C, Diniz T, Patarra RF, Álvaro NV, Neto AI (2013) Coccolithophore species as indicators of surface oceanographic conditions in the vicinity of Azores islands. *Estuarine, Coastal and Shelf Science* 118: 50-59.

Smith ADM, Brown CJ, Bulman CM, Fulton EA, Johnson P, Kaplan IC, Lozano-Montes H, Mackinson S, Marzloff M, Shannon LJ, Shin YJ, Tam J (2011) Impacts of fishing low-trophic level species on marine ecosystems. *Science* 333: 1147-1150.

Sponaugle S, Cowen RK, Shanks A, Morgan SG, Leis JM, Pineda J, Boehlert GW, Kingsford MJ, Lindeman KC, Grimes C, Munro JL (2002) Predicting self-recruitment in marine populations: Biophysical correlates and mechanisms. *Bulletin of Marine Science* 70: 341-375.

Swain DP, Sinclair AF, Hanson JM (2007) Evolutionary response to size-selective mortality in an exploited fish population. *Proceedings of the Royal Society of London B: Biological Sciences* 274: 1015-1022.

Swearer SE, Caselle JE, Lea DW, Warner RR (1999) Larval retention and recruitment in an island population of a coral-reef fish. *Nature* 402: 799-802

Vale M (2016) *Influence of climate change and other impacts on rocky intertidal communities of the Azores.* PhD thesis, University of Southampton.

Walsh MR, Munch SB, Chiba S, Conover DO (2006) Maladaptive changes in multiple traits caused by fishing: impediments to population recovery. *Ecology Letters* 9: 142-148.

Weber LI, Hawkins SJ (2005) *Patella aspera* and *P. ulyssiponensis*: genetic evidence of speciation in the North-east Atlantic. *Marine Biology* 147: 153-162.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* 163: 1177-1191.

Wright S (1943) Isolation by distance. *Genetics* 28: 114-138.

Zhivotovsky LA (2015) Relationships between Writht's F_{ST} and F_{IS} statistics in a context of Wahlund effect. *Journal of Heredity* 106: 306-309.

SUPPLEMENTARY MATERIAL

Methods

2.1. Sampling and laboratory procedures

Briefly, 17 microsatellite markers were amplified in three multiplex PCR reactions containing 5 – 6 loci using the Qiagen® PCR multiplexing kit according to the manufacturer's recommendations. Amplifications took place on a MyCycler™ thermal cycler (BioRad) using a touchdown protocol and a final volume of 10 µL with approximately 10 ng DNA template, 1 × Qiagen™ Multiplex PCR Kit, 0.4 - 1.2 µM of each primer and ddH_2O. The PCR routine for each multiplex reaction consisted of an initial denaturation step at 95°C for 15 min; 5 cycles of touchdown amplification (denaturation at 94°C for 30 sec, annealing temperature (T_A) (see Faria *et al.* 2015) for 1 min descending by 0.5°C intervals to the optimal T_A, plus extension at 72°C for 40 sec; 22 cycles at 94°C for 30 sec, optimal T_A for 1 min, and extension at 72°C for 40 sec; 10 cycles at 94°C for 30 sec, 53°C for 50 sec, 72°C for 45 sec; and a final extension step at 60°C for 30 min. Approximately 20% of the samples were re-run to ensure repeatability in scoring. Amplified fragments were visualized using an ABI 3730 (Applied Biosystems) automated DNA sequencer with GeneScan™ 500Liz (Applied Biosystems) as an internal size standard for accurate sizing. Genotypes were called using GENEMAPPER™ v.4.2 (Applied Biosystems).

2.2. Genetic diversity

LOSITAN (Antao *et al.* 2008): LOSITAN applies the F_{ST} outlier detection approach to identify loci potentially under selection. In order to avoid potential bias when assigning F_{ST} as outlier due to random drift and historical events affecting gene flow, the two more geographically distant populations (Madeira and Gran Canaria) were removed from analysis.

2.3. Population structure

POWSIM v.4.0 (Ryman and Palm 2006): Simulations were carried out considering combinations of several effective population sizes (N_e) and number of generations (t). Observed allele frequencies were used as a starting point. In all cases, 1 000 replicates were run and the power of the analysis was indicated by the proportion of tests that were significant at $P < 0.05$ based on chi-square and Fisher's exact tests.

STRUCTURE v.2.3.4 (Pritchard *et al.* 2000): STRUCTURE, which allows determining the most plausible K number of genetically homogeneous groups in the dataset. The analyses were run with 10^5 burn-in iterations followed by 5×10^5 Markov chain Monte Carlo steps, applying an admixture ancestry model with correlated allele frequencies and prior sampling location information (LOCPRIOR

= 1). Considering the origin of individuals as prior information is more powerful in detecting weak genetic structure, reduces mis-assignments and increases the sensibility of STRUCTURE in clustering populations with low genetic divergence (Hubisz *et al.* 2009).

GENELAND (Guillot *et al.* 2005): GENELAND which can account for the presence of null alleles while incorporating geographical coordinates information for genotyped individuals to estimate the most likely number of clusters and their spatial boundaries. Given the prior knowledge regarding dispersal and potential barriers to gene flow in *P. aspera*, preference was given to the spatial model with null alleles. Even so, distinct model conditions were also tested. To improve the detection of subtle genetic structure (Guillot 2008), all models assumed correlated allele frequencies. For each model, three independent MCMC runs were conducted, each consisting of 2×10^7 iterations with a thinning of 1 000 after a burn-in of 2×10^3. Consistency between runs was assessed by checking that they provided approximately the same parameter estimates (K, individual population membership, maps). The highest average posterior probability was used as a criterion to select the best run under the given set of model conditions.

2.5. Bottleneck detection and gene flow

BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996): Because microsatellites rarely conform strictly to the infinite allele mutation (IAM) model or the stepwise-mutation model (SMM), the two-phase model (TPM) accommodating both mutation types was adopted (Di Rienzo *et al.* 1994; Piry *et al.* 1999). TPM was parameterized with 78% and 90% single-step mutations, assuming a conservative variance among multiple steps of 12 as recommended for microsatellites (Piry *et al.* 1999, Peery *et al.* 2012). The significance of the heterozygosity excess was assessed using the one-tail Wilcoxon sign-rank test with 10 000 iterations. Each analysis was independently carried out for each inferred genetic cluster provided by STRUCTURE and GENELAND (see Results).

M_p_val (Garza and Williamson 2001): This analysis is considered to be more appropriate to detect genetic bottlenecks that occurred over relatively long periods of time (> 100 generations). The M-ratio test is based on the ratio of the number of alleles over the range in allele size and it can identify gaps in the allele size frequency distribution resulting from loss of alleles due to bottleneck events. Loci with odd-sized alleles (those that did not represent multiples of the recognized repeat unit) were omitted from analyses. Three parameters are required by the method: the pre-bottleneck value of $\Theta = 4N_e mu$ (where N_e is the effective pre-bottleneck population size at equilibrium and *mu* is the mutation rate), the fraction of mutations that are larger than single steps (*ps*) and the average size of non-single-step mutations (Δg). Because such information is not available for *P. aspera* in particular, a broad range of input parameters were tested, including Garza and Williamson (2001) findings and recommendations (see Results). A range of values of Θ from 0.1 to 200 (corresponding to *Ne* values of 50 - 100 000) were tested to determine the sensitivity of M to changes in such parameter and assuming an average mutation rate of 5×10^{-4}. The observed M-ratio for each cluster was then compared to the critical value obtained in CRITICAL_M and estimated through 10 000 simulations with the same parameters as the data and assuming the population to be at drift–migration equilibrium. Lower M-ratios than

populations at equilibrium is taken as evidence that the sample is from a population that had experienced a recent bottleneck (Garza and Williamson 2001).

BAYESASS v.3.0 (Wilson and Rannala 2003): Ten MCMC runs with different initial seeds using 10^6 burn-in, following by 3×10^6 sampling iterations were carried out in order to maximize convergence and mixing. Mixing parameters were adjusted to achieve acceptance rates of 20 - 40%. The Bayesian deviance was used as an optimality criterion to find the run with the best fit (Faubet *et al.* 2007). Deviance was calculated from the trace file using the R-script provided by Meirmans (2014).

References

Antao T, Lopes A, Lopes RJ, Beja-Pereira A, Luikart G (2008) LOSITAN: A workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics* 9: 323.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144: 2001-2014.

Di Rienzo A, Peterson AC, Garcza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple-sequence repeat loci in human populations. *Proceedings of the National Academy of Sciences of the USA* 91: 3166-3170.

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46: 96-97.

Faubet P, Waples RS, Gaggiotti OE (2007) Evaluating the performance of a multilocus Bayesian method for the estimation of migration rates. *Molecular Ecology* 16: 1149-1166

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* 10: 305-318.

Guillot G (2008) Inference of structure in subdivided populations at low levels of genetic differentiation - the correlated allele frequencies model revisited. *Bioinformatics* 24: 2222-2228.

Guillot G, Mortier F, Estoup A (2005) GENELAND: a program for landscape genetics. *Molecular Ecology Resources* 5: 712-715.

Hubisz MJ, Falush D, Stephens M, Pritchard JK (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources* 9: 1322-1332

Meirmans PG (2014) Non-convergence in Bayesian estimation of migration rates. *Molecular Ecology Resources* 14: 726-733.

Peery MZ, Kirby R, Reid BN, Stoelting R, Doucet-Bêer E, Robinson S, Vásquez-Carrillo C, Pauli JN, Palsboll PJ (2012) Reliability of genetic bottleneck tests for detecting recent population declines. *Molecular Ecology* 21: 3403-3418.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* 90: 502-503.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Ryman N, Palm S (2006) POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes* 6: 600-602.

Wilson GA, Rannala B (2003) Bayesian inference of recent migration rates using multilocus genotypes. *Genetics* 163: 1177-1191.

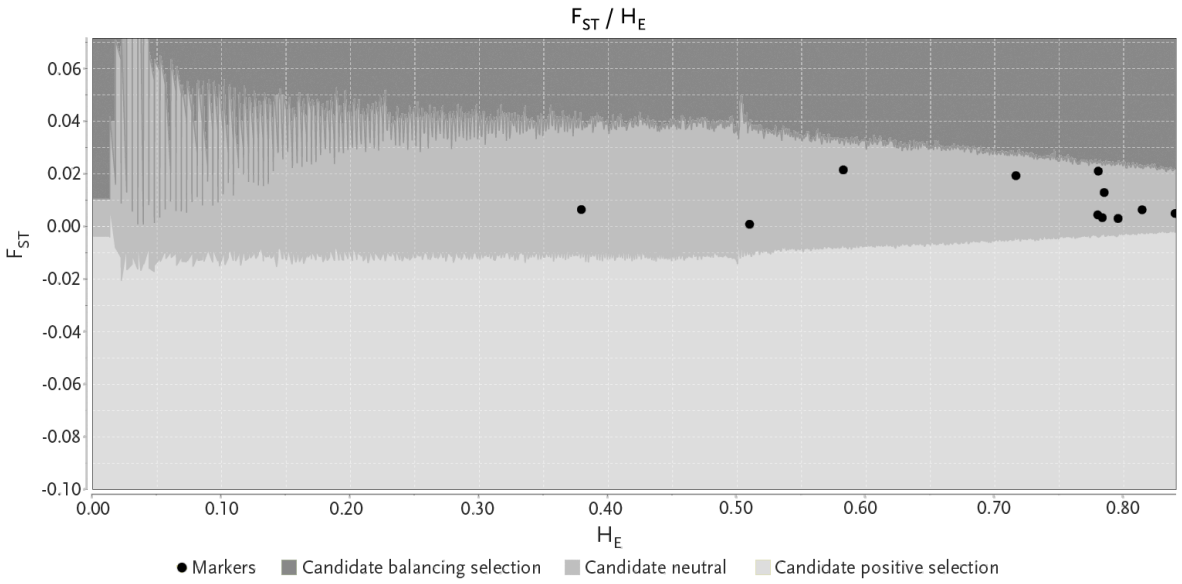


Figure S1. Test for selection on 11 microsatellite loci in *Patella aspera* using LOSITAN. Black circles represent loci within neutral expectations.

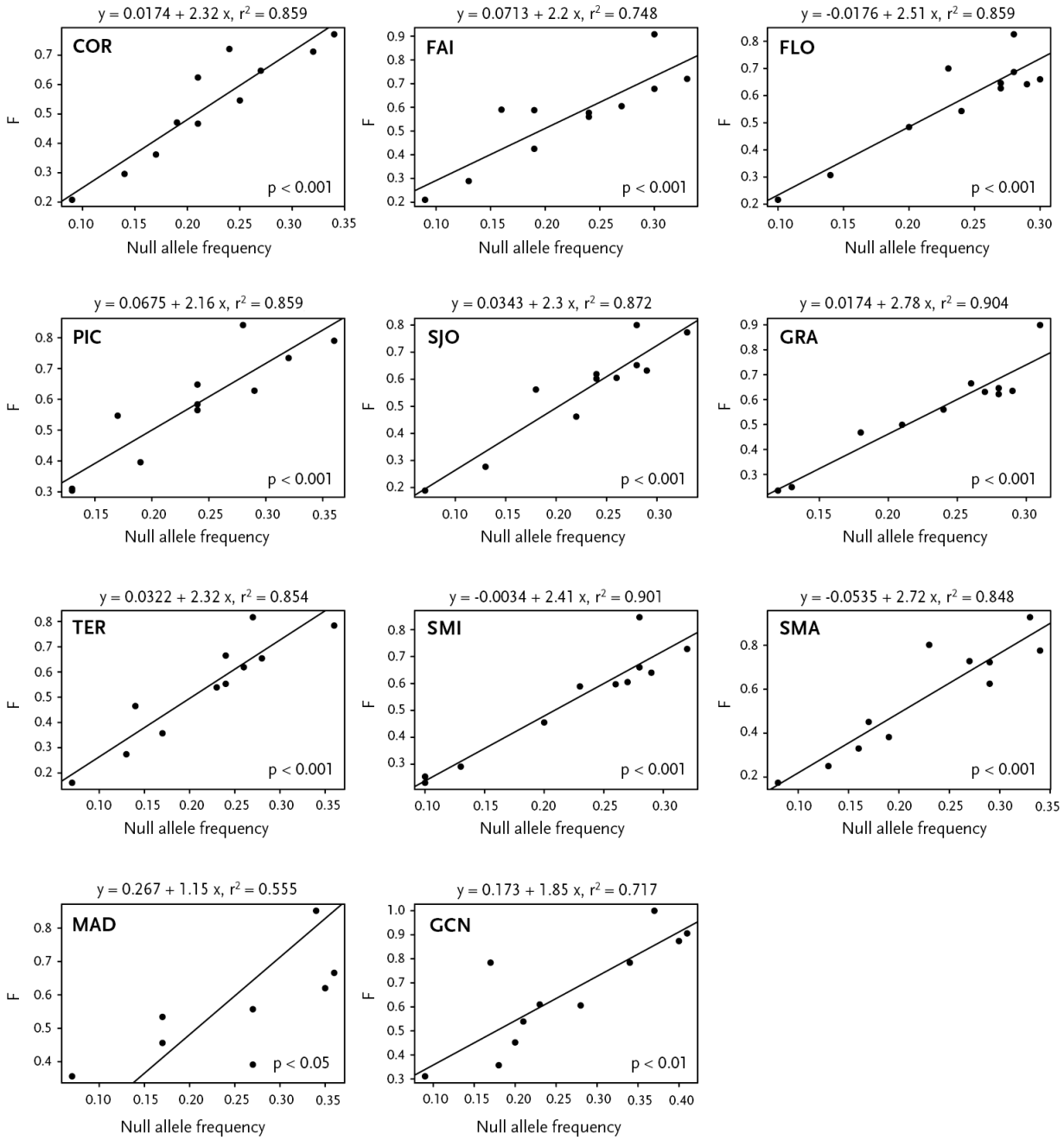


Figure S2. Regression analyses between uncorrected F_{IS} and frequency of null alleles for all locations sampled (see Fig. 1 for population codes).

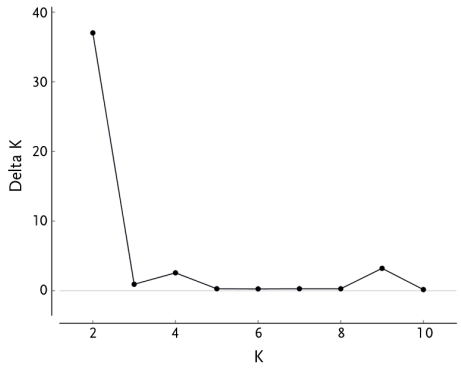


Figure S3. Estimated ΔK for the STRUCTURE analysis considering an admixture ancestry model with correlated allele frequencies and prior sampling location information.

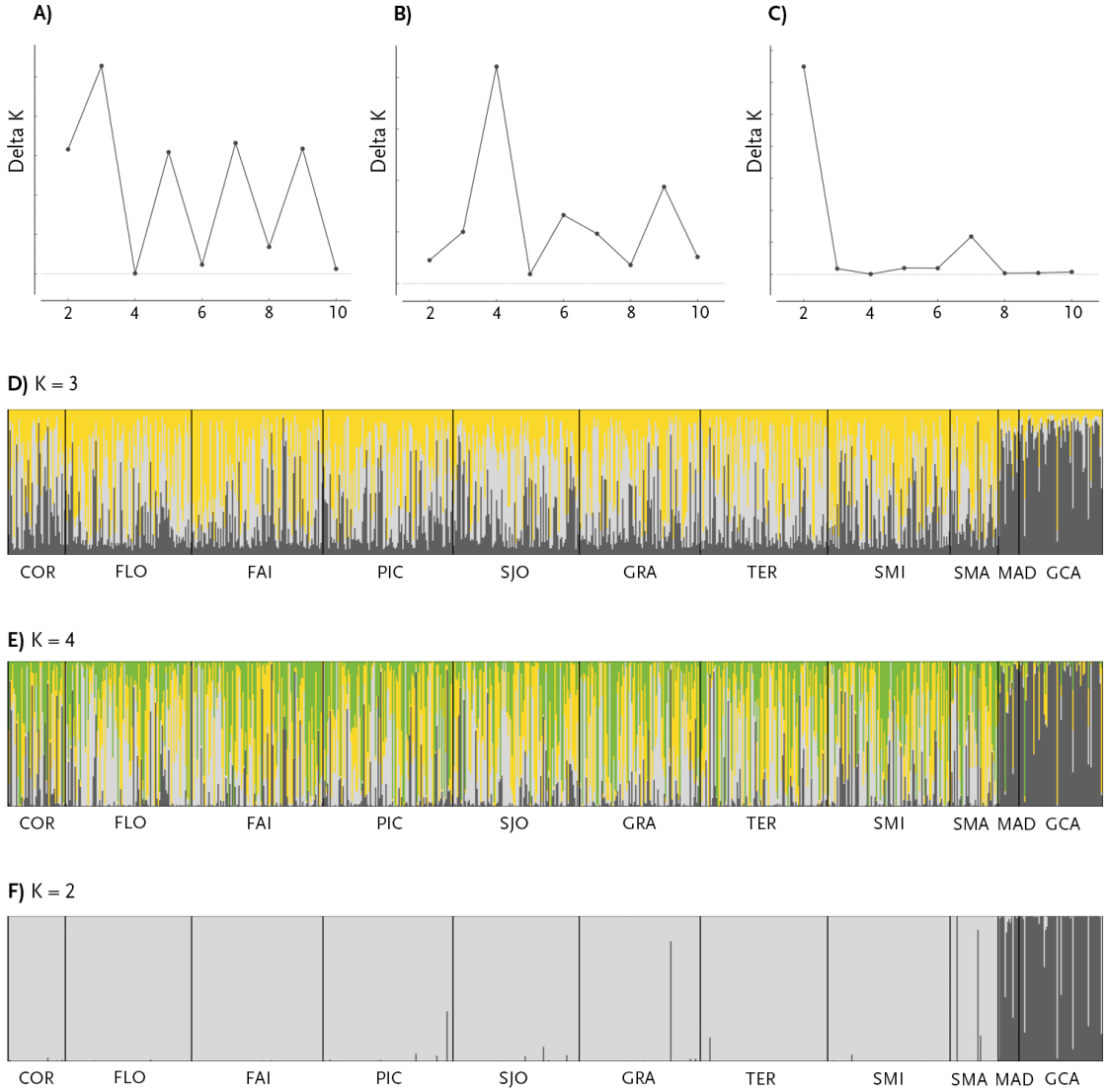


Figure S4. ΔK and STRUCTURE analyses results for *Patella aspera* using 11 loci considering the admixture ancestry model without prior information for population origin (A and D, respectively); no admixture ancestry model without prior information for population origin (B and E, respectively); and no admixture ancestry model with prior information for population origin (C and F, respectively). See Fig. 1 for population codes).

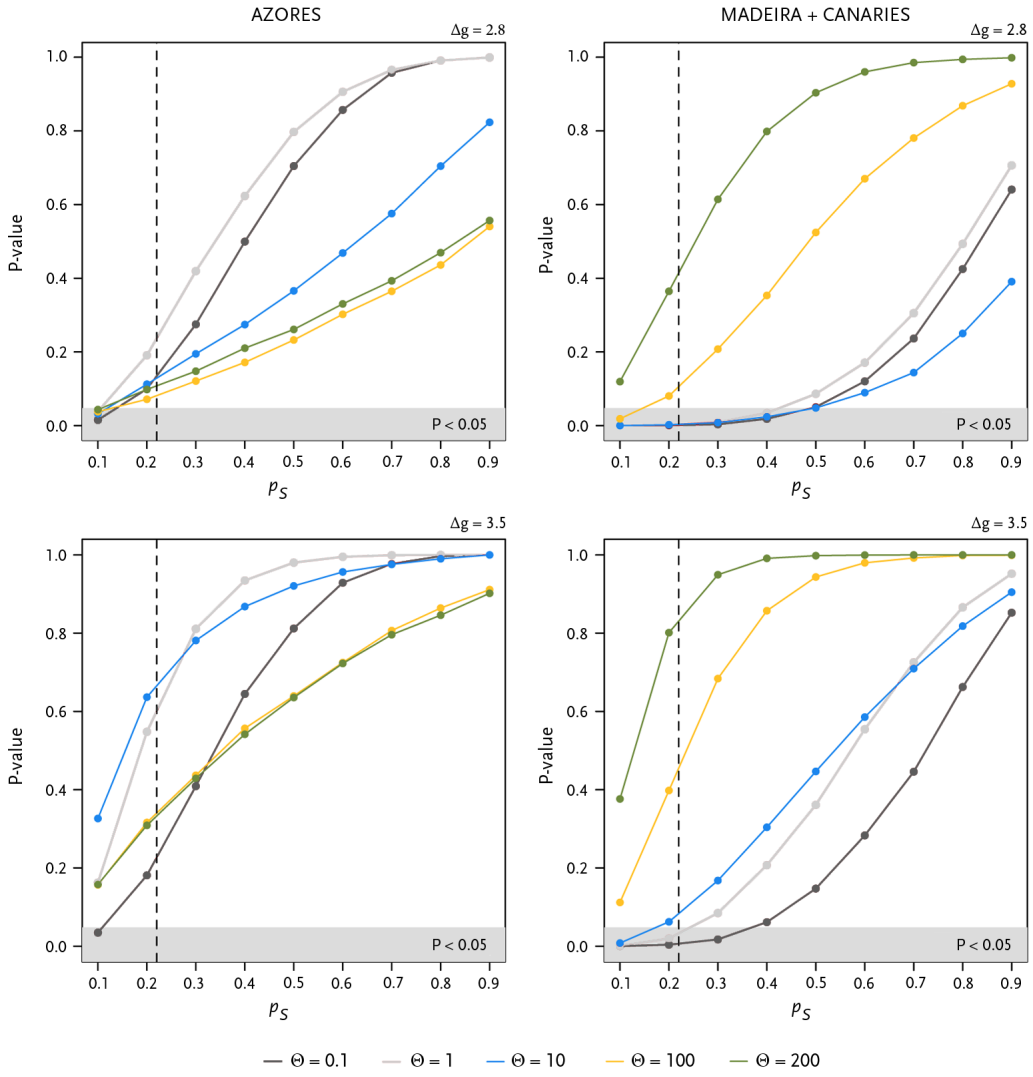


Figure S5. Bottleneck M-ratio detection analyses in *Patella aspera* for Δg = 2.8 (top graphs) and Δg = 3.5 (bottom graphs) considering Θ = 0.1 to 200 and p_S = 0.1 to 0.9. Dots in the grey area (P < 0.05) indicate bottleneck detection under the specified settings.

Table S1. Genetic variation observed at eleven microsatellite loci within eleven populations sampled for *Patella aspera* (see Fig. 1 for population codes).

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
N	44	97	101	100	97	93	98	94	37	16	64	841
ASP2												
N_A	6	9	11	10	9	9	8	8	7	7	12	20
$A_R(10)$	4.5	4.7	4.8	4.7	4.4	4.7	4.7	4.4	4.6	4.9	6.2	-
$A_P(10)$	0.0	0.2	0.3	0.3	0.1	0.2	0.1	0.2	0.2	0.6	1.6	-
H_O	0.628	0.632	0.630	0.550	0.629	0.609	0.660	0.582	0.556	0.286	0.078	0.531
H_E	0.793	0.799	0.804	0.790	0.775	0.796	0.787	0.780	0.741	0.753	0.831	0.786
F_{IS}	0.219	0.215	0.228	0.298	0.181	0.259	0.170	0.232	0.311	0.818	0.891	
Null	0.09	0.09	0.10	0.13	0.07	0.12	0.07	0.10	0.13	0.35	0.41	0.15[a]
ASP3												
N_A	8	7	8	7	7	9	6	8	6	4	9	10
$A_R(10)$	4.7	2.6	4.3	3.4	3.9	4.1	3.2	3.9	3.1	6.3	4.6	
$A_P(10)$	0.2	0.1	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.5	0.2	
H_O	0.256	0.149	0.214	0.191	0.240	0.215	0.159	0.247	0.034	0.091	0.100	0.172
H_E	0.724	0.361	0.685	0.543	0.629	0.641	0.475	0.602	0.479	0.616	0.794	0.595
F_{IS}	0.654	0.593	0.680	0.651	0.635	0.633	0.700	0.594	1.000	0.761	0.901	
Null	0.27	0.19	0.28	0.24	0.24	0.26	0.24	0.23	0.33	0.34	0.40	0.27
ASP7												
N_A	3	2	3	5	5	3	3	4	5	2	2	14
$A_R(10)$	2.1	2.0	2.1	2.3	2.5	2.2	2.0	2.1	2.7	-	6.6	
$A_P(10)$	0.1	0.0	0.0	0.1	0.3	0.2	0.0	0.1	0.3	-	5.1	
H_O	0.140	0.045	0.088	0.079	0.110	0.050	0.090	0.076	0.156	0.000	0.000	0.076
H_E	0.500	0.494	0.505	0.495	0.550	0.495	0.491	0.491	0.574	0.245	0.500	0.485
F_{IS}	0.727	0.909	0.828	0.840	0.804	0.901	0.815	0.843	0.755	-	0.833	
Null	0.24	0.30	0.28	0.28	0.28	0.31	0.27	0.28	0.27	-	0.37	0.29
ASP17												
N_A	8	8	8	9	8	8	9	9	7	6	8	15
$A_R(10)$	5.2	4.7	4.6	4.8	4.5	4.6	4.6	4.6	4.7	3.0	3.9	
$A_P(10)$	0.2	0.1	0.1	0.2	0.2	0.1	0.2	0.2	0.3	0.0	0.6	
H_O	0.523	0.253	0.350	0.206	0.172	0.270	0.255	0.311	0.618	0.267	0.220	0.313
H_E	0.819	0.785	0.766	0.775	0.759	0.761	0.737	0.772	0.747	0.798	0.565	0.753
F_{IS}	0.372	0.681	0.552	0.737	0.763	0.652	0.659	0.598	0.179	0.906	0.593	
Null	0.17	0.30	0.24	0.32	0.33	0.28	0.28	0.26	0.08	0.36	0.23	0.26
ASP21												
N_A	9	10	11	10	11	10	11	9	9	6	11	18
$A_R(10)$	4.6	5.0	4.7	4.5	4.7	4.8	5.3	4.5	4.7	4.2	3.7	
$A_P(10)$	0.2	0.2	0.2	0.1	0.2	0.2	0.3	0.2	0.2	0.9	0.2	
H_O	0.535	0.562	0.540	0.520	0.563	0.589	0.598	0.578	0.500	0.333	0.458	0.525
H_E	0.760	0.790	0.779	0.754	0.778	0.784	0.824	0.752	0.747	0.715	0.664	0.759
F_{IS}	0.307	0.294	0.317	0.302	0.274	0.277	0.283	0.222	0.378	0.427	0.228	
Null	0.14	0.13	0.14	0.13	0.13	0.13	0.13	0.10	0.16	0.17	0.09	0.13[a]

continue

continued

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
ASP27												
N_A	10	8	12	8	9	7	8	9	8	4	10	21
$A_R(10)$	4.2	4.2	4.7	4.2	4.1	4.0	4.2	4.9	4.4	3.0	4.2	
$A_P(10)$	0.4	0.1	0.3	0.1	0.1	0.1	0.2	0.4	0.6	0.0	0.3	
H_O	0.386	0.333	0.293	0.333	0.256	0.326	0.351	0.284	0.429	0.429	0.167	0.326
H_E	0.730	0.758	0.785	0.766	0.736	0.741	0.762	0.787	0.780	0.704	0.773	0.757
F_{IS}	0.480	0.564	0.621	0.567	0.660	0.572	0.538	0.650	0.394	0.750	0.796	
Null	0.19	0.24	0.27	0.24	0.28	0.24	0.23	0.29	0.17	0.27	0.34	0.25
ASP29												
N_A	12	22	24	16	15	17	15	15	12	6	20	44
$A_R(10)$	5.5	6.2	5.9	5.2	5.7	5.4	5.9	5.5	6.3	6.0	5.6	
$A_P(10)$	0.4	0.9	1.0	0.4	0.4	0.4	0.6	0.3	0.5	2.3	1.6	
H_O	0.235	0.239	0.284	0.167	0.306	0.292	0.183	0.324	0.192	0.333	0.306	0.260
H_E	0.818	0.855	0.836	0.792	0.832	0.800	0.850	0.820	0.859	0.753	0.777	0.817
F_{IS}	0.720	0.723	0.663	0.806	0.641	0.661	0.788	0.612	0.752	0.621	0.628	
Null	0.32	0.33	0.30	0.36	0.29	0.29	0.36	0.27	0.34	0.27	0.28	0.31
ASP33												
N_A	11	17	13	14	16	13	14	11	15	10	16	22
$A_R(10)$	5.5	5.1	4.2	5.4	5.8	4.9	5.2	5.0	6.1	5.9	6.5	
$A_P(10)$	0.4	0.3	0.3	0.4	0.4	0.2	0.3	0.1	0.7	0.7	1.4	
H_O	0.429	0.438	0.312	0.475	0.447	0.374	0.500	0.420	0.500	0.545	0.559	0.454
H_E	0.805	0.761	0.604	0.786	0.830	0.746	0.778	0.772	0.809	0.847	0.870	0.783
F_{IS}	0.477	0.430	0.477	0.400	0.487	0.486	0.369	0.459	0.417	0.197	0.394	
Null	0.21	0.19	0.20	0.19	0.22	0.21	0.17	0.20	0.19	0.07	0.18	0.18[a]
ASP36												
N_A	13	13	15	12	12	13	11	15	9	6	11	22
$A_R(10)$	5.9	5.3	5.6	5.1	5.2	5.3	4.8	5.1	4.8	-	4.8	
$A_P(10)$	0.6	0.3	0.4	0.2	0.2	0.5	0.3	0.6	0.5	-	0.6	
H_O	0.386	0.321	0.297	0.293	0.318	0.300	0.346	0.218	0.276	0.333	0.421	0.319
H_E	0.851	0.813	0.829	0.788	0.804	0.795	0.773	0.801	0.735	0.750	0.769	0.792
F_{IS}	0.554	0.609	0.636	0.650	0.598	0.640	0.549	0.722	0.661	-	0.475	
Null	0.25	0.27	0.29	0.29	0.26	0.28	0.24	0.32	0.29	-	0.20	0.27
ASP38												
N_A	11	8	13	8	7	12	10	10	6	4	10	22
$A_R(10)$	5.1	3.7	4.4	3.9	3.6	4.6	4.0	4.1	4.2	3.0	4.5	
$A_P(10)$	0.9	0.1	0.5	0.2	0.1	0.4	0.2	0.2	0.2	1.0	1.1	
H_O	0.182	0.266	0.250	0.265	0.250	0.261	0.276	0.247	0.200	0.400	0.333	0.266
H_E	0.794	0.629	0.707	0.638	0.628	0.707	0.722	0.728	0.721	0.735	0.724	0.703
F_{IS}	0.776	0.581	0.645	0.588	0.594	0.638	0.616	0.676	0.680	0.467	0.516	
Null	0.34	0.24	0.27	0.24	0.24	0.27	0.26	0.28	0.29	0.17	0.21	0.25

continue

continued

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA	All pops.
ASP39												
N_A	9	4	6	7	7	9	6	8	5	3	3	13
$A_R(10)$	3.3	2.0	2.5	2.7	2.5	2.9	2.5	2.5	2.9	-	1.8	
$A_P(10)$	0.9	0.1	0.2	0.3	0.2	0.4	0.1	0.3	0.1	-	0.0	
H_O	0.182	0.104	0.116	0.165	0.160	0.221	0.186	0.198	0.086	0.286	0.038	0.158
H_E	0.483	0.254	0.386	0.364	0.364	0.415	0.347	0.279	0.432	0.582	0.178	0.371
F_{IS}	0.631	0.595	0.713	0.525	0.556	0.509	0.445	0.375	0.709	-	0.792	
Null	0.21	0.16	0.23	0.17	0.18	0.18	0.14	0.13	0.23	-	0.17	0.18[a]
Multilocus												
Mean $A_R(10)$	4.9	4.5	4.7	4.5	4.6	4.6	4.6	4.6	4.8	4.5	4.9	
Mean $A_P(10)$	0.4	0.2	0.4	0.2	0.2	0.3	0.2	0.3	0.3	0.8	1.3	
Mean H_O	0.353	0.304	0.397	0.295	0.314	0.319	0.328	0.317	0.322	0.300	0.244	0.309
Mean H_E	0.734	0.663	0.699	0.681	0.699	0.698	0.686	0.689	0.693	0.682	0.677	0.691
$F_{IS\text{-}INEST}$	0.481	0.389	0.497	0.428	0.409	0.441	0.399	0.188	0.393	0.179	0.113	0.342

N = number of samples; N_A = number of alleles; $A_R(g)$ = allelic richness (g accounts for the maximum standardized sample size i.e. twice the number of genotypes); $A_P(g)$ = private allelic richness; H_O = observed heterozygosity; H_E = unbiased expected heterozygosity; F_{IS} = uncorrected inbreeding coefficient; $F_{IS\text{-}INEST}$ = "null free" inbreeding coefficient; a = loci used on restricted dataset analysis.

Table S2. Significance of differences (P-value) from Wilcoxon signed rank test for allelic richness A_R (below diagonal) and private allelic richness A_P (above diagonal) between locations (see Fig. 1 for population codes).

	COR	FLO	FAI	PIC	SJO	GRA	TER	SMI	SMA	MAD	GCA
COR	-	0.382	0.721	0.505	0.382	0.645	0.721	0.505	0.879	0.227	0.050
FLO	0.574	-	0.161	0.505	0.879	0.834	0.442	0.721	0.442	0.189	0.021
FAI	0.505	0.879	-	0.328	0.235	0.235	0.442	0.279	0.721	0.293	0.130
PIC	0.328	1.000	0.879	-	0.574	0.798	0.721	0.879	0.505	0.103	0.021
SJO	0.328	0.798	0.574	0.959	-	0.959	0.574	0.959	0.328	0.103	0.021
GRA	0.442	0.959	0.959	0.798	0.645	-	0.382	1.000	0.328	0.103	0.015
TER	0.721	0.959	1.000	0.879	0.798	1.000	-	0.645	0.798	0.128	0.050
SMI	0.235	0.879	0.959	0.879	0.721	0.879	0.798	-	0.505	0.103	0.021
SMA	0.721	0.959	0.879	0.879	0.505	0.959	0.879	0.798	-	0.270	0.065
MAD	0.798	1.000	1.000	1.000	0.959	0.959	1.000	1.000	0.574	-	0.563
GCA	0.879	0.879	0.959	0.645	0.574	1.000	0.798	0.721	0.959	0.574	-

Table S3. Analysis of molecular variance (AMOVA) among the two *Patella aspera* clusters derived from STRUCTURE and GENELAND analyses.

Source of variation	Variation (%)	Fixation indices
Among clusters	8.2	0.092***
Among populations within clusters	0.9	0.011***
Within populations	90.8	0.022***

*** P < 0.001

Table S4. Deviance Information Criterion (DIC) scores provided by INEST for the null model (a model including genotyping error and inbreeding: nb) and the full model (a model including genotyping error, inbreeding and null alleles: nfb) (see Fig. 1 for population codes).

Model	nb	nfb
COR	2736.274	2708.248
FLO	5312.890	5300.481
FAI	5868.074	5811.853
PIC	5496.800	5466.992
SJO	5398.970	5380.678
GRA	5370.465	5342.176
TER	5357.972	5326.156
SMI	5208.252	5190.103
SMA	2164.283	2153.798
MAD	624.098	622.409
GCA	3344.047	3342.533
All populations	49174.890	48987.208
Clusters		
AZORES	43599.466	43441.136
MADEIRA+CANARIES	4115.341	4112.377

Table S5. Analysis of recent genetic bottlenecks in *Patella aspera* estimated for 4 microsatellite loci (loci with null allele occurrence were not included) in 2 clusters derived from STRUCTURE and GENELAND analyses using the sign-rank Wilcoxon test of the mutation–drift equilibrium under a two-phased model of mutation (TPM) parameterized with 78% and 90% single-step mutations.

Cluster	Mean sample size	Mean number of alleles	Mean H_e	Probability TPM 78%	Probability TPM 90%
AZORES	1397.1	17.9	0.701	1.000	0.989
MADEIRA + CANARIES	105.6	11.3	0.727	0.991	0.938

Null hypothesis: $H_e = H_{eq}$ - population at mutation-drift equilibrium; H_1: $H_e > H_{eq}$ - excess gene diversity indicating recent bottleneck

Table S6. Contemporary gene flow in *Patella aspera* between the 2 clusters derived from STRUCTURE and GENELAND analyses, given by the means of the posterior distributions of migration rates calculated in BAYESASS (and 95% CI).

From/into	AZORES	MADEIRA + CANARIES
AZORES	0.999 (0.997-1.000)	0.001 (0.000-0.003)
MADEIRA + CANARIES	0.020 (0.000-0.044)	0.980 (0.956-1.000)

CHAPTER 6

Spatial and temporal patterns in recruitment in the exploited limpet *Patella candei*

ABSTRACT

The rate of input of new individuals is a key driver of population dynamics. When considering marine exploited species population persistence is a fine balance between individuals that are removed by harvesting and individuals that are added to the population via recruitment. A spatially and temporally replicated monitoring programme was put in place during 2014-2015 to assess the levels of recruitment of the exploited limpet *Patella candei* (d'Orbigny 1839) in the Azores (NE Atlantic). Recruitment occurred throughout both years but its intensity varied in space and time. The mean number of recruits per m^2 in 2014 and 2015 was 26.3 (±1.7 SE) and 11.3 (±0.9 SE), respectively. In general, a marked peak in recruitment occurred during winter/spring months, the period of greatest reproductive activity, when sea surface temperatures were lower and wave turbulence higher. There was a significant correlation between sea surface temperature (SST), significant wave height (H_S), wave period (T_Z) and wave direction (W_{DIR}) and limpet recruitment. While each of these environmental variables may have individually influenced reproduction and recruitment, significant wave height (H_S) was probably the most important proximate factor triggering the recruitment of *P. candei*. This single variable accounted for the observed inter-annual variation in recruitment levels. The understanding of how such environmental processes are coupled with larval recruitment dynamics is a key aspect in fisheries science, and may help to improve conservation strategies aimed at achieving a sustainable use of exploited marine resources.

KEYWORDS: settlement, environmental variables, wave action, dispersal

Manuscript in preparation as:

Faria J, Ribeiro PA, Miller P, Azevedo ED, Hawkins SJ, Neto AI, Martins GM. *In prep*. Spatial and temporal patterns in recruitment in the exploited limpet *Patella candei*.

1. Introduction

Recruitment, which is broadly regarded as the addition of new individuals to populations, is considered a critical step for marine organisms to thrive (Caley *et al.* 1996; Palumbi and Pinsky 2014). Many of these organisms are sessile or sedentary as adults; therefore their persistence is largely or exclusively dependent on the addition of new juveniles to the population. If this compensatory mechanism is somehow reduced, then populations will be particularly vulnerable to over-harvesting or other perturbations (Fogarty *et al.* 1991). Conversely, impacts on marine adult populations (e.g. over-exploitation) can negatively impact their reproductive output, fertilization success and hence subsequent recruitment (e.g. Kelly *et al.* 2009). Local production of offspring may, however, have little to no direct effect in determining population size, providing that connectivity allows replenishing of populations by the addition of individuals produced elsewhere (Underwood and Fairweather 1989; Caley *et al.* 1996).

In most marine benthic invertebrate species, reproduction occurs through the release of eggs or gamete into the water and by developing a planktonic larval stage that can potentially disperse tens to hundreds of kilometers in the water column before settling into suitable habitats. The development, survival and settlement of these early-life stages are dependent on the combined forces of several physical and biological factors, such as temperature (e.g. Han 2011; Rankin and Sponaugle 2011), wave action (e.g. Jenkins *et al.* 1997), light conditions (Tatsumi and Wright 2016), food availability (e.g. Castonguay *et al.* 2008), competition (e.g. Hixon and Jones 2005) and predation (e.g. Doherty *et al.* 2004). Moreover, many larvae exhibit selective settlement (e.g. Knight-Jones 1953; Crisp 1974; Jenkins 2005), mediated by physiological and behavioural responses to physical or chemical cues broadly associated with adult presence at suitable sites (e.g. Crisp 1967; Crisp and Meadows 1962; Miron *et al.* 1996; Kay 2002; Cahill and Koury 2016), or by habitat complexity (e.g. Underwood 2004; Bohn *et al.* 2013) or biofilms (Thompson *et al.* 1998). The challenges associated with the addition of new individuals via pelagic larvae to sessile and often patchy adult populations are immense and critical for the persistence and dynamics of marine benthic species. Larval stages are thus the most sensitive stage in the life cycle of many marine benthic organisms. Furthermore, post-settlement processes involving complex interactions between predators (Keough and Downes 1982; Doherty *et al.* 2004), competitors (e.g. Denley and Underwood 1979; Underwood *et al.* 1983), shelter (Butler and Herrnkind 1997) and food availability, and physical disturbances (Lewis and Bowman 1975) can have a major influence on recruitment success (see review by Hunt and Scheibling 1997). Understanding the scale at which physical and biological processes are coupled with larval recruitment has a crucial importance in identifying temporal abundance patterns of adult populations, their distribution and resilience to disturbances. This information can then be used in implementing marine ecosystem management and conservation approaches to marine stocks facing continuous exploitation.

The limpet *Patella candei* (d'Orbigny 1840) is a marine gastropod mollusc that commonly occurs at mid to high intertidal levels on rocky shores across the Macaronesia archipelagos (NE Atlantic) with the exception of Cape Verde. As a broadcast spawner, *P. candei* releases gametes into the water column, and upon fertilization, they go thorough a planktonic larval stage wherein a trochophore larvae

develops to a pre and post-torsional veliger, before settling on hard substrate and metamorphosing. As in other patellids, the pelagic larval duration is assumed to last between a few days and few weeks, depending on water temperature (Hawkins *et al.* 2000; Ribeiro *et al.* 2009). The success of fertilization is dependent of a number of variables (e.g. sperm concentration, gamete age and contact time) but these are not likely to significantly affect dispersal, which is mostly a function of the duration of the planktonic larvae in the water column (Hodgson *et al.* 2007). According to Martins *et al.* (1987), *P. candei* shows no marked peak of spawning and resting period throughout the year. Yet, more recent studies have confirmed that *P. candei* is in fact primarily a winter breeder (see Henriques *et al.* 2012; Vale 2016), with a greater proportion of individuals reaching the spawning stage (see gonad ripening stages in Orton *et al.* 1956) between late-autumn and spring months, with a resting season in the summer. In *Patella vulgata* (Linnaeus 1758), winter breeding is generally assumed to be associated with a decrease in temperature (Bowman and Lewis 1977) and/or to more turbulent water conditions (Blackmore 1969). Whereas temperature can play a vital role in reproductive phenology and early larval growth-related traits, wave action is considered to trigger spawning and facilitate the release and transport of gametes in *Patella* (mainly studies on *P. vulgata*: Orton *et al.* 1956; Bowman and Lewis 1977, 1986; Thompson 1979; Moore *et al.* 2011).

The fate of *P. candei* larvae in their natural environment is largely unknown. Despite living in a pelagic environment structured by physical processes, which may limit the species capacity to disperse, patellid larvae are offered a vast range of opportunities that may help them find a suitable settlement site. Recent evidence from in-vitro fertilization trials showed that *P. candei* larvae are planktotrophic and can feed on diatoms (see Chapter 7). Planktotrophic larvae have the potential to disperse longer distances (Scheltema 1986). Moreover, patellid trochophore and veliger larvae are active swimmers, showing bursts of fast upward/horizontal swimming and passive sinking (Ribeiro 2008), which may allow them to position in the water column in areas where food is available and/or currents are favourable. Such conspicuous behaviour exposes larvae to different flow regimes, which is likely to affect and determine their growth, mortality and dispersal (e.g. Fiksen *et al.* 2007). Patellid larvae also show chemotaxic behaviour upon substratum selection (Ribeiro 2008). This allows individuals to find the most suitable habitat, providing higher chances of survival and post-settlement performance (Elkin and Marshall 2007). For instance, the presence of crustose coralline algae is known to induce substratum-specific settlement, attachment and metamorphosis in several marine gastropods including abalones (Morse *et al.* 1984; Barlow 1990; Roberts and Nicholson 1997) and limpets (McGrath and Foley 2005; Ribeiro 2008). The exact cues for such site selection are still poorly understood.

Patellid larvae may also be challenged in finding suitable space to settle on rocky shores. Long-term exploitation of limpets in the Azores (Martins *et al.* 2008a) has resulted in most of the space at mid-shore levels being dominated by algal turfs, which preclude the settlement of limpets (Martins *et al.* 2010). This has also been shown elsewhere (see Underwood *et al.* 1983; Boaventura *et al.* 2002).

Very little information is available on recruitment of *P. candei*, and most of it comes from anecdotal evidence derived from field observations or extrapolations made from adult gonad maturation periods

(Vale 2016) and length–frequency observations (Henriques *et al.* 2012). This study is aimed at quantifying the temporal and spatial variation in recruitment of *P. candei* on the south coast of São Miguel Island, Azores, and establish possible relationships between real-time environmental data such as sea surface temperatures and wave action, and limpet recruitment. This information is not only crucial for understanding the population dynamics of exploited stocks of *P. candei*, but also to assess the role of physical/environmental processes in recruitment variability.

2. Methods

2.1. Study sites and communities

This study was conducted on two consecutive years (2014 - 2015) across stretches of 20 m in four locations of the São Miguel Island, Azores (Fig. 1). All locations are of volcanic origin (basalt *sensu lato*) and moderately exposed to southerly wave action, and consist of small rocky platforms that support similar assemblages of animals and plants. The lower shore is mostly dominated by coarsely branched algae (e.g. articulated coralline algae, *Gelidium* spp. and *Caulacanthus ustulatus*), whilst the barnacle *Chthamalus stellatus* dominates on the upper shore. Ephemeral algae such as *Ulva* spp. and *Chaetomorpha* spp. can be seasonally abundant and scattered clumps of the brown algae *Fucus spiralis* are also observed at mid-shore levels. Common fauna include the periwinkles *Tectarius striatus* and *Melarhaphe neritoides*, and the patellid species *P. candei*, which are the most abundant littoral grazers (Hawkins *et al.* 1990, Martins *et al.* 2008b).

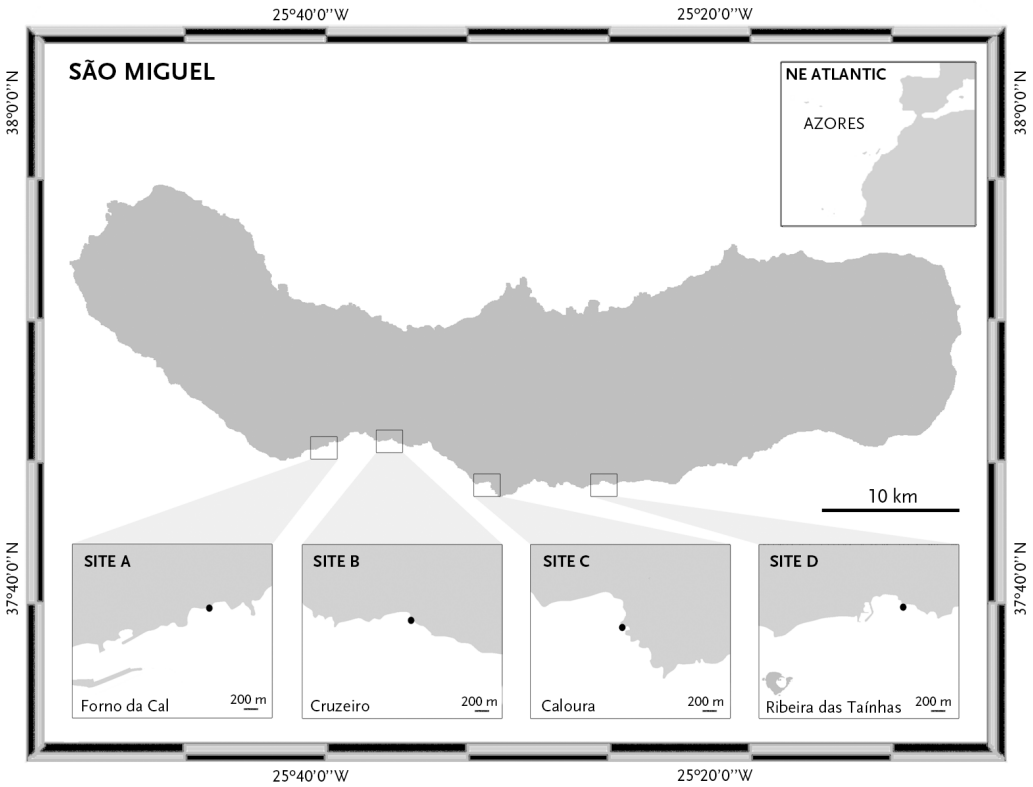


Figure 1. Sampling locations in São Miguel island, Azores.

2.2. Sampling design

A total of 15 basaltic plates (7 × 7 cm) mimicking the natural coastal substrate, were initially placed at the mid-shore level in the transition between algal turfs and barnacles at each location, where *P. candei* abundance is highest (Martins *et al.* 2010). These plates were collected monthly during spring low tides and replaced by new ones. Quantification of *P. candei* recruits on plates was done under an optical microscope. Individuals with 0.5 cm or less (total shell length) were considered as recruits (or recently recruited) as in Martins *et al.* (2010) (Fig. S1). As in other patellids (e.g. Hartnoll and Wright 1977), *P. candei* also exhibits a homing behaviour (E. Cacabelos, unpublished data 2014), where individuals upon recruiting in a small rock depression/ hole/ crevice to which the shell margin conforms, tend to return to the same area after foraging excursions. Such homing behaviour on top of our size-based definition of recruits ensures that the number of smaller-sized animals observed on a given plate are likely a surrogate of recent settlement leading to recruitment in that particular month.

Environmental data were obtained from a moored buoy located on the south coast of São Miguel island, Azores (37º 43.546' N / 25º 43.284') which is run under the projects CLIMAAT - MAC 2.3/A.3, CLIMAAT II - 03/MAC/2.3/A.5 and CLIMARCOST – 05/MAC/2.3/A1. Sea surface temperature (SST), significant wave height (H_S), wave period (T_Z) and wave direction (W_{DIR}: degrees north from which the waves are traveling) were recorded at time intervals of 10 secs during the course of this study. Due to missing data (owing to buoy malfunction during part of the experimental period), SST estimates were also obtained from the Jet Propulsion Laboratory's Multi-Scale High Resolution SST (MUR SST), which merges data from infrared and passive microwave datasets collected from satellites into global daily maps gridded at 1 km resolution, generating a product that contains no cloud contamination (see Chin *et al.* 1998; more information can be found at http://mur.jpl.nasa.gov/). SST data were extracted on a weekly basis around a 2 km buffer of the sampling locations. Monthly time series were reconstructed for all environmental variables.

2.3. Data analysis

Spatial and temporal differences in limpet recruitment were examined using a 3-way analysis of variance (ANOVA) with the following factors: 'year' (fixed factor, 2 levels), 'month' (random factor and nested within year, 12 levels) and 'location' (random factor and orthogonal to other factors, 4 levels) with 15 replicates. Temporal variation in environmental variables was analysed by means of a 2-way ANOVA following the same general design but without the factor 'location'.

Given that recruitment of *P. candei* showed a clear peak during early months in each year (see section below), analyses were also run using a subset of months corresponding to this specific period and thus avoiding the influence of an excessive number of zeros in the analyses. This recruitment period was defined as the broadest time interval where monthly recruitment averages were greater than the yearly average, which corresponded, in this study, to the period between January and April. All tests were done using PERMANOVA+ based on Euclidean distances and using 999 permutations (Anderson *et al.* 2008). Prior to analyses, data were checked for heterogeneity of variances and

transformations were applied when needed (Underwood 1997). Analyses were performed on untransformed data when attempts to stabilize variances through transformation were unsuccessful. In such cases, because of the increased likelihood of Type I error, a more conservative significance level ($\alpha = 0.01$) was used when testing for differences (Underwood 1997).

The association between recruitment and each environmental variable was assessed in R v.3.3.0 (R Core Team 2014) using a correlation/ linear regression analysis. Due to high collinearity among environmental variables (see Quinn and Keough 2002), the distance-based linear models routine (DistLM, Anderson *et al.* 2008) was then used to examine how much of the variability in *P. candei* recruitment was accounted for by each of the environmental (predictor) variables measured by the buoy. Predictor variables were fitted individually in marginal tests to determine their relationship with recruitment and identify their relative contribution for the observed variation in recruitment. All DistLM analyses were based on Euclidean dissimilarities, and P-values for testing the null hypothesis of no relationship were obtained using 999 permutations.

3. Results

Although recruitment of *P. candei* was recorded throughout both years of survey, its intensity varied in space and time (Fig. 2; Table S1; Fig. S2). The mean number of recruits per m^2 in 2014 and 2015 was 26.3 (± 1.7 SE) and 11.3 (± 0.9 SE), respectively. A marked peak in recruitment occurred in winter/spring months, especially in 2014 when recruitment was substantially greater (Fig. 3). When considering only the period of highest recruitment (January to April), analyses showed a significant interaction between 'year' and 'location' (ANOVA, $P < 0.05$; Table S2). Inspection of a posteriori tests showed that recruitment of *P. candei* was consistently higher in 2014 on all four locations examined (Fig. 4). During this period, the mean number of recruits per m^2 in 2014 and 2015 was 57 (± 3.9 SE) and 17.1 (± 2.0 SE), respectively.

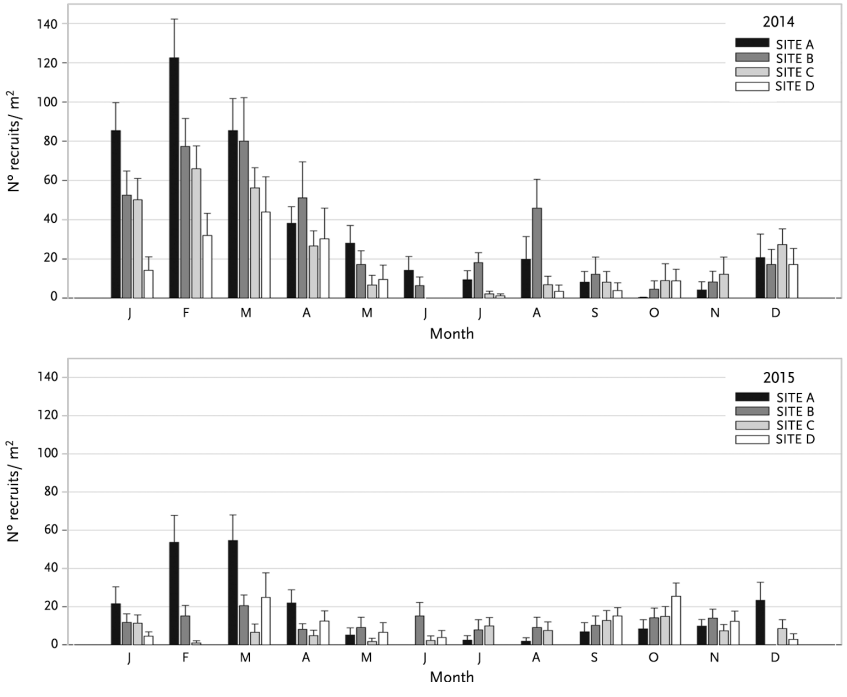


Figure 2. Recruitment variation of *Patella candei* during 2014 (top) and 2015 (bottom) in 4 locations in São Miguel island.

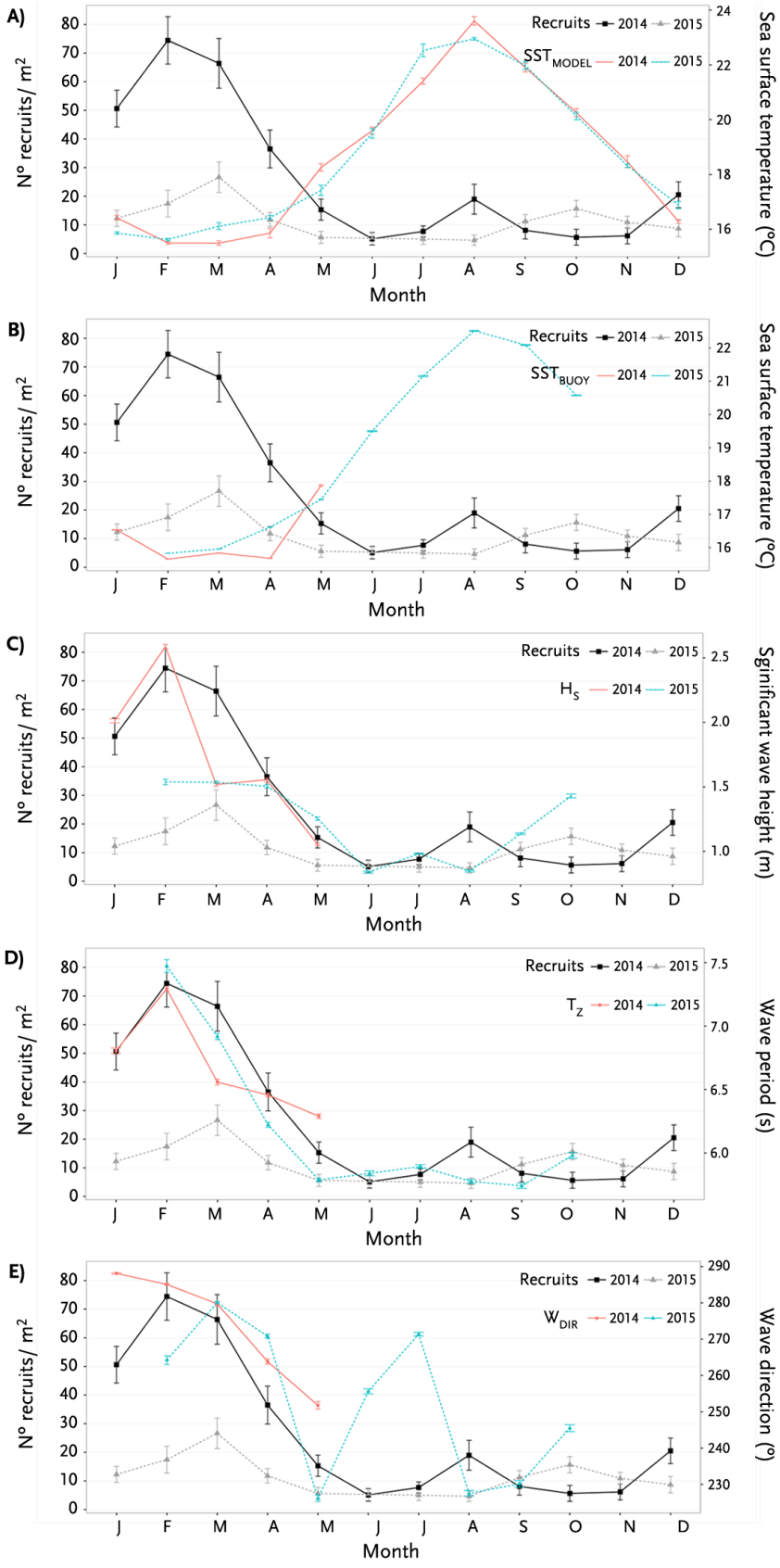


Figure 3. Mean number of *Patella candei* recruits during 2014 and 2015 and monthly records for the environmental variables A) sea surface temperature - SST_{MODEL}; B) sea surface temperature - SST_{BUOY}, C) significant wave height (H_S), D) wave period (T_Z) and E) wave direction (W_{DIR}).

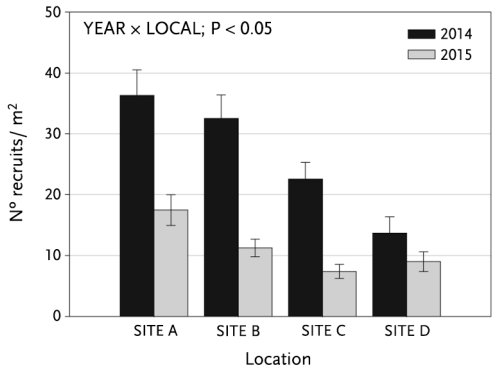


Figure 4. Mean number of *Patella candei* recruits in each location during January to April on 2014 and 2015. All pairwise comparisons on term YEAR × LOCATION for pairs of levels of factor YEAR are significantly different (P < 0.05).

There was a significant correlation between all the environmental variables and recruitment (Fig. S3). Whilst the relationship between recruitment and SSTs was negative, the relationship between recruitment and the remaining variables was positive (Fig. S3). The DistLM analysis showed that environmental variables, when considered separately, significantly predicted the recruitment of *P. candei*. However, marginal tests (Table 1) showed that H_S was the best single predictor accounting for about 69% of the variation in recruitment, whilst W_{DIR}, T_Z and SST_{BUOY} explained a reduced proportion of recruitment (48%, 44% and 40% respectively). Moreover, significant variation between years for the environmental variables was only detected for wave height (ANOVA, P < 0.05; Table S3), which was 51.3 % higher in 2014 compared to 2015 (15.1% when considering only the same months in both years).

Table 1. Marginal tests results for the relationship between *Patella candei* recruitment and the predictor variables SST_{BUOY} – sea surface temperature retrieved from buoy, H_S – significant wave height, T_Z – wave period and W_{DIR} – wave direction. Variation %: percentage of variance in recruitment explained by environmental variables.

	Variation %	Pseudo-F
H_S	0.692	26.983***
W_{DIR}	0.480	11.060**
T_Z	0.435	9.245*
SST_{BUOY}	0.399	7.979*

* P < 0.05, ** P < 0.01, *** P < 0.001

4. Discussion

This study shows that *P. candei* recruitment, although occurring throughout the year, shows a peak during winter/spring months (January to April), following peak reproductive activity when sea surfaces temperatures are lowest and wave action is highest. A similar pattern was described for *P. candei* in Madeira Island, about 920 km southeast of Azores (see Henriques *et al.* 2012). While each of the four environmental variables investigated here may have influenced recruitment on its own, significant

wave height (H_S) was probably the most important factor triggering the recruitment of *P. candei.* In fact, it was the only variable that effectively accounted for the observed inter-annual variation in recruitment: a roughly 40% decrease in limpet recruitment from 2014 to 2015. Wave action has been positively associated with release (Orton *et al.* 1956; Orton and Southward 1961; Shanks 1998) the transport and settlement of larvae on coastal shores (Jeffery and Underwood 2000) and it is considered a good proxy of nearshore larval transport. This relationship, however, is likely to follow a dome-shaped function (see Cury and Roy 1989), in which the capacity of larvae to settle and recruit may be limited by higher wave weights (Ortega 1981). Yet, as with any conclusions based on correlations, this strong relationship between recruitment variation and wave height can be caused by other covarying factors such as temperature, wind speeds, wind direction, water flow, tides, coastal upwellings and/or nutrient inputs. These and many other processes often interact together in determining recruitment variation at multiple spatial and temporal scales (see Pineda *et al.* 2007, 2009). For instance, the spatial variation detected in *P. candei* recruitment likely reflects the intricate influence of several spatially structured nearshore processes operating at very localized spatial scales i.e. without ruling out stochastic events by themselves, features such as local geomorphology (Palma *et al.* 2006), substrate microtopography (Kohler *et al.* 1999) and local flow patterns (Archambault and Bourget 1999; Shanks *et al.* 2003) may interact in a complex way to determine if recruits are to occur or not in precise rocky shores areas, leading to unpredictable patterns of spatial variability in recruitment. Moreover, the spatial variability in recruitment may arise from processes that directly affect settlement and post-settlement mortality rates, such as the larval response to various abiotic and biotic cues on the substratum, including surface texture or chemistry, conspecifics, and the presence or absence of other macrobenthic species or microbial biofilms (see review in Hunt and Scheibling 1997). Interestingly, the trend in recruitment levels of *P. candei*, although variable between sites, showed a remarkable consistency between years (see Fig. 4). This highlights the significant importance of small spatial-scale processes in determining local coastal communities.

The life-history strategy of *P. candei* seems to take advantage of wintertime conditions as a means to maximize reproduction, spawning, larval development, dispersal and survival. But the magnitude and interplay of conditions that affect the outcome of any one of these phases can be considerably different. For instance, temperature is known to be an ultimate driver for phenology and reproductive traits such as maturing and the development of ripe-stage gonads in limpets, while storms and wave action are often seen as proximate cues to spawning events (Orton *et al.* 1956; Bowman and Lewis 1977; Moore *et al.* 2011). The variable nature of such processes will ultimately play a determinant role in shaping recruitment variability patterns in space and time. For instance, as homologous temperatures did not differ between years, the most likely cause for recruitment failure in 2015 in *P. candei* may be associated to inadequate conditions of processes that usually work as proximate cues to spawning and/or recruitment, such has storms and wave action.

As winter brooders, the temperatures recorded during winter/spring months are probably within the range for optimal phenology, larval development and early recruit growth in *P. candei*. Also, within the North Atlantic subtropical province, where Azores is included, wintertime mixing provides the seasonal replenishment of nutrients to the euphotic zone while in spring, thermal stratification favours

phytoplankton growth, which progressively leads to surface nutrient depletion by late summer (Silva *et al.* 2013). Assuming that the phytoplankton is the primary food source for *P. candei* larvae, the most beneficial timing for reproduction and recruitment would be prior or overlaid to the period of highest food abundance, providing that there is a positive balance between food gains and predatory and competition losses. Moreover, the life-history strategy of *P. candei* allows the species to avoid the time of the year when coastal thermal stress is higher and wave action is weaker, preventing mortality of early recruits from desiccation or overheating conditions to which the early stages of many marine invertebrates are susceptible (e.g. Kordas *et al.* 2015).

Due to the geographical isolation of the Azores, recruitment is restricted to the local production of offspring and/or dispersal from more distant populations within the archipelago (Faria *et al.* 2017). Identifying the exact source of recruits remains a great challenge for marine ecologists, especially because of the difficulties in tracking such small size individuals through the dynamic nature of the pelagic environment (Swearer *et al.* 2002). Nevertheless, a growing number of studies indicate that self-recruitment is a common process in many benthic marine species (e.g. Jones *et al.* 1999; Kingsford *et al.* 2002; Teske *et al.* 2016), providing selective advantages over long-distance dispersal (see Strathmann *et al.* 2002). In fact, for many marine species, larval behaviour and nearshore hydrodynamic processes may promote the retention of planktonic larvae close to parental habitats. In such cases, the ability of larvae to recruit back to their natal populations may offer them a more effective opportunity of finding suitable habitats and minimize the risk of predation (Swearer *et al.* 2002). Despite this, there is no solid indication that self-recruitment is the main process acting upon *P. candei* populations. Whether this might be the case, several aspects of the life history of *P. candei* seem to support a more open population model, with larvae dispersing over long distances and settling far from their parents' area, on occasional fast recruitment events during storms. First, feeding larvae have the potential to support a relatively long pelagic duration and, despite their swimming capacity, *P. candei* larvae are quite small and likely act as passive particles that can be easily transported by currents to more remote locations far from their origin i.e. between close-by islands in Azores. Secondly, most *P. candei* gametes are released into the water column during wintertime (Vale 2016), under stronger turbulent waters, which promote mixing, fertilization and initial transport away from parental areas. Moreover, in São Miguel island, local recruitment does not seem to be strictly correlated with local production i.e. November has the highest percentage of ripe individuals (Vale 2016) and recruitment tends to peak between January and April. As mentioned above, this temporal mismatch can probably be a consequence of specific environmental requirements needed to trigger reproduction and recruitment, and does not necessarily mean that recruits are originating from elsewhere; ripe gonads do occur throughout the winter/spring season and given the appropriate cues for spawning (Vale 2016), recruitment can still be generated locally.

Another aspect to note is derived from stock-recruitment relationships. For instance, Martins *et al.* (2008a) showed that the number of islanders per coastal perimeter is a good predictor of the abundance of *P. candei* across the Azorean archipelago, and that the number of larger animals decrease with increasing island population. Being the most populated island, São Miguel has presumably the lowest number of larger individuals of *P. candei* per coastal perimeter, considerably

limiting the reproductive capacity and offspring production of the species on the island. Therefore, a likely source of *P. candei* recruits in São Miguel could be from close-by populations from different islands of the archipelago (i.e. Santa Maria, Formigas). As long as recruitment continues, these highly exploited populations will persist even if local production is limited. Although the implications of genetic connectivity and demographic connectivity can be substantially different (see Lowe and Allendorf 2010), the homogeneous gene pool detected throughout the archipelago (Faria *et al.* 2017) may further support this open population model, where connectivity between limpet populations in different islands is maintained via exchange of pelagic larvae.

Although many physical and biological processes are known to play a pivotal role in the development and survival of larval stages, settlers and early recruits in marine invertebrates, the direct effect of each process is hardly identifiable. Despite the challenges, understanding how these processes are coupled with larval recruitment has crucial importance to implement marine ecosystem management and conservation initiatives. Moreover, the prediction of recruitment fluctuations, especially considering the environmental changes that are expected under a rapid climate change scenario (i.e. warmer oceans, extreme weather events; IPCC 2014) is a fundamental aspect to consider in assessing metapopulation dynamics and resilience in exploited marine organisms.

Acknowledgments

We thank Afonso Prestes, Cristina Bernabeu, Eva Cacabelos, Ignatio Moreu, Isadora Moniz, Maria del Mar, Maria Vale, Marina Prieto, Marta Coca, Sophia Griese and Zaira Nogueira for helping in fieldwork and/or recruitment counts. JF was supported through a PhD grant (M3.1.2/ F/021/2011) funded by the Regional Government of the Azores.

References

Anderson MJ, Gorley RN, Clarke KR (2008) *PERMANOVA for PRIMER: guide to software and statistical methods*. PRIMER-E Ltd., Plymouth, United Kingdom.

Archambault P, Bourget E (1999) Influence of shoreline configuration on spatial variation of meroplanktonic larvae, recruitment and diversity of benthic subtidal communities. *Journal of Experimental Marine Biology and Ecology* 238: 161-184.

Barlow LA (1990) Electrophysiological and behavioral responses of larvae of the red abalone *(Haliotis rufescens)* to settlement-inducing substances. *Bulletin of Marine Science* 46: 537-554.

Blackmore DT (1969) Studies on *Patella vulgata* L. I. Growth, reproduction and zonal distribution. *Journal of Experimental Marine Biology and Ecology* 3: 200-213.

Boaventura D, Alexander M, Santina PD, Smith ND, Ré P, Fonseca LC, Hawkins SJ (2002) The effects of grazing on the distribution and composition of low-shore algal communities on the

central coast of Portugal and on the southern coast of Britain. *Journal of Experimental Marine Ecology and Biology 2*67: 185-206.

Bohn K, Richardson CA, Jenkins SR (2013) Larval microhabitat associations of the non-native gastropod *Crepidula fornicata* and effects on recruitment success in the intertidal zone. *Journal of Experimental Marine Biology and Ecology* 448: 289-297.

Bowman RS, Lewis JR (1977) Annual fluctuations in the recruitment of *Patella vulgata* L. *Journal of the Marine Biological Association of the UK* 57: 793-815.

Bowman RS, Lewis JR (1986) Geographical variation in the breeding cycles and recruitment of *Patella* spp. *Hydrobiologia* 142: 41-56.

Butler MJ, Herrnkind WF (1997) A test of recruitment limitation and the potential for artificial enhancement of spiny lobster (*Panulirus argus*) populations in Florida. *Canadian Journal of Fisheries and Aquatic Sciences* 54: 452-463.

Cahill AE, Koury SA (2016) Larval settlement and metamorphosis in a marine gastropod in response to multiple conspecific cues. *PeerJ* 4:e2295.

Caley MJ, Carr MH, Hixon MA, Hughes TP, Jones GP, Menge BA (1996) Recruitment and the local dynamics of open marine populations. *Annual Review of Ecology and Systematics* 27: 477-500.

Castonguay M, Plourde S, Robert D, Runge JA, Fortier L (2008) Copepod production drives recruitment in a marine fish. *Canadian Journal of Fisheries and Aquatic Sciences* 65: 1528-1531.

Chin TM, Milliff RF, Large WG (1998) Basin-scale, high-wavenumber sea surface wind fields from a multiresolution analysis of scatterometer data. *Journal of Atmospheric and Oceanic Technology* 15: 741-763.

Crisp DJ (1967) Chemical factors inducing settlement in *Crassostrea virginica* (Gmelin). *Journal of Animal Ecology* 36: 329-335.

Crisp DJ (1974) Factors influencing the settlement of marine invertebrate larvae. In *Chemoreception in marine organisms.* Grant PT, Mackie AM (Eds). Academic Press, London, pp. 177-265.

Crisp DJ, Meadows PS (1962) The chemical basis of gregariousness in cirripedes. *Proceedings of the Royal Society of London B - Biological sciences* 156: 500-520.

Cury P, Roy C (1989) Optimal environmental window and pelagic fish recruitment success in upwelling areas. *Canadian Journal of Fisheries and Aquatic Sciences* 46: 670-680.

Denley EJ, Underwood AJ (1979) Experiments on factors influencing settlement, survival, and growth of two species of barnacles in New South Wales. *Journal of Experimental Marine Biology and Ecology* 36: 269-293.

Doherty PJ, Dufour V, Galzin R, Hixon MA, Meekan MG, Planes S (2004) High Mortality during settlement is a population bottleneck for a tropical surgeonfish. *Ecology* 85: 2422-2428.

Elkin C, Marshall DJ (2007) Desperate larvae: influence of deferred costs and habitat requirements on habitat selection. *Marine Ecology Progress Series* 335: 143-153.

Faria J, Martins GM, Pita A, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017) Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic). *Ecology and Evolution* 7(16): 6125-6140.

Fiksen O, Jørgensen C, Kristiansen T, Vikebø F, Huse G (2007) Linking behavioural ecology and oceanography: larval behaviour determines growth, mortality and dispersal. *Marine Ecology Progress Series* 347: 195-205.

Fogarty MJ, Sissenwine MP, Cohen EB (1991) Recruitment variability and the dynamics of exploited marine populations. *Trends in Ecology and Evolution* 6: 241-246.

Han YS (2011) Temperature-dependent recruitment delay of the Japanese glass eel *Anguilla japonica* in East Asia. *Marine Biology* 158: 2349-2358.

Hartnoll RG, Wright JR (1977) Foraging movements and homing in the limpet *Patella vulgata* L. *Animal Behaviour* 25: 806-810.

Hawkins SJ, Burnay LP, Neto AI, Da Cunha RT, Maritns AMF (1990) A description of the zonation patterns of molluscs and other important biota on the south coast of São Miguel, Azores. *Açoreana* (Suppl.): 21-38.

Hawkins SJ, Côrte-Real HBSM, Pannacciulli FG, Weber LC, Bishop JDD (2000) Thoughts on the ecology and evolution of the intertidal biota of the Azores and other Atlantic islands. *Hydrobiologia* 440: 3-17.

Henriques P, Sousa R, Pinto AR, Delgado J, Faria G, Alves A, Khaden M (2012) Life history traits of the exploited limpet *Patella candei* (Mollusca: Patellogastropoda) of the north-eastern Atlantic. *Journal of the Marine Biological Association of the UK* 92: 1379-1387.

Hixon MA, Jones GP (2005) Competition, predation, and density-dependent mortality in demersal marine fishes. *Ecology* 86: 2847-2859.

Hodgson AN, Quesne W, Hawkins SJ, Bishop JDD (2007) Factors affecting fertilization success in two species of patellid limpet (Mollusca: Gastropoda) and development of fertilization kinetics models. *Marine Biology* 150: 415-526.

Hunt HL, Scheibling RE (1997) Role of early post-settlement mortality in recruitment of benthic marine invertebrates. *Marine Ecology Progress Series* 155: 269-301.

IPCC (2014) *Climate Change 2014: Synthesis Report*. Contribution of Working Groups I, II and III to the Fifth assessment report of the intergovernmental panel on climate change [Core Writing Team, RK Pachauri, LA Meyer (Eds)]. IPCC, Geneva, Switzerland.

Jeffery CJ, Underwood AJ (2000) Consistent spatial patterns of arrival of larvae of the honeycomb barnacle *Chamaesipho tasmanica* Foster and Anderson in New South Wales. *Journal of Experimental Marine Biology and Ecology* 252: 109-127.

Jenkins GP, Black KP, Wheatley MJ, Hatton DN (1997) Temporal and spatial variability in recruitment of a temperate, seagrass-associated fish is largely determined by physical processes in the pre- and post-settlement phases. *Marine Ecology Progress Series* 148: 23-35.

Jenkins SR (2005) Larval habitat selection, not larval supply, determines settlement patterns and adult distribution in two chthamalid barnacles. *Journal of Animal Ecology* 74: 893-904.

Jones GP, Millicich MJ, Emslie MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. *Nature* 402: 802-804.

Kay MC (2002) Recruitment in the intertidal limpet *Lottia digitalis* (Patellogastropoda: Lottiidae) may be driven by settlement cues associated with adult habitat. *Marine Biology* 141: 467-477.

Kelly JE, Frank KT, Leggett WC (2009) Degraded recruitment synchrony in Northwest Atlantic cod stocks. *Marine Ecology Progress Series* 393: 131-146.

Keough MJ, Downes BJ (1982) Recruitment of marine invertebrates: the role of active larval choices and early mortality. *Oecologia* 54: 348-352.

Kingsford MJ, Leis JM, Shanks A, Lindeman KC, Morgan SG, Pineda J (2002) Sensory environments, larval abilities and local self-recruitment. *Bulletin of Marine Science* 70: 309-340.

Knight-Jones EW (1953) Laboratory experiments on gregariousness during setting in *Balanus balanoides* and other barnacles. *Journal of Experimental Biology* 30: 584-598.

Kohler J, Hansen PD, Wahl M (1999) Colonization patterns at the substratum-water interface: how does surface microtopography influence recruitment patterns of sessile organisms?. *Biofouling* 14: 237-248.

Kordas RL, Dudgeon S, Storey S, Harley CDG (2015) Intertidal community responses to field-based experimental warming. *Oikos* 124: 888-898.

Lewis JR, Bowman RS (1975) Local habitat-induced variations in the population dynamics of *Patella vulgata* L. *Journal of Experimental Marine Biology and Ecology* 17: 165-203.

Lowe WH, Allendorf FW (2010) What can genetics tell us about population connectivity?. *Molecular Ecology* 19: 3038-3051.

Martins HR, Santos RS, Hawkins SJ (1987) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008a) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Thompson RC, Hawkins SJ, Neto AI, Jenkins SR (2008b) Rocky intertidal community structure in oceanic islands: scales of spatial variability. *Marine Ecology Progress Series* 356: 15-24.

Martins GM, Thompson RC, Neto AI, Hawkins SJ, Jenkins SR (2010) Exploitation of intertidal grazers as a driver of community divergence. *Journal of Applied Ecology* 47: 1282-1289.

McGrath D, Foley H (2005) Settlement and recruitment of the blue-rayed limpet, *Patella pellucida* L. in Galway Bay, west coast of Ireland. In *The intertidal ecosystem: the value of Ireland's shores.* Wilson JG (Ed). Royal Irish Academy, Dublin. pp. 100-114.

Miron G, Bourget E, Archambault P (1996) Scale of observation and distribution of adult conspecifics: their influence in assessing passive and active settlement mechanisms in the barnacle *Balanus crenatus* (Brugiere). *Journal of Experimental Marine Biology and Ecology* 201: 137-158.

Moore PJ, Thompson RC, Hawkins SJ (2011) Phenological changes in intertidal conspecific gastropods in response to climate warming. *Global Change Biology* 17: 709-719.

Morse ANC, Froyd CA, Morse DE (1984) Molecules from cyanobacteria and red algae that induce settlement and metamorphosis in the mollusc *Haliotis rufescens*. *Marine Biology* 81: 293-298.

Ortega S (1981) Environmental stress, competition and dominance of *Crasstostrea virginica* near Beaufort, North Carolina, USA. *Marine Biology* 62: 47-56.

Orton JH, Southward AJ (1961) Studies on the biology of limpets IV. The breeding of *Patella depressa* Pennant on the north Cornish coast. *Journal of the Marine Biological Association of the UK* 41: 653-662.

Orton JH, Southward AJ, Dodd JM (1956) Studies on the biology of limpets. II. The breeding of *Patella vulgata* L. in Britain. *Journal of the Marine Biological Association of the UK* 35: 149-176.

Palma, AT, Pardo LM, Veas R, Cartes C, Silva M, Manriquez K, Diaz A, Muñoz C, Ojeda FP (2006) Coastal brachyuran decapods: settlement and recruitment under contrasting coastal geometry conditions. *Marine Ecology Progress Series* 316: 139-153.

Palumbi SR, Pinsky ML (2014) Marine dispersal, ecology, and conservation. In *Marine community ecology and conservation.* Bertness MD, Bruno JF, Silliman BR, Stachowicz JJ (Eds). Sinauer, Sunderland, USA. pp. 57-83.

Pineda J, Hare JA, Sponaugle S (2007) Larval dispersal and transport in the coastal ocean and consequences for population connectivity. *Oceanography* 20: 22-39.

Pineda J, Reyns NB, Starczak VR (2009) Complexity and simplification in understanding recruitment in benthic populations. *Population Ecology* 51: 17-32.

Quinn G, Keough M (2002) *Experimental design and data analysis for biologists*. Cambridge University Press, Cambridge.

R Core Team (2014) R: *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available from: http://www.R-project.org/.

Rankin TL, Sponaugle S (2011) Temperature influences selective mortality during the early life stages of a coral reef fish. *PLoS ONE* 6, e16814.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Ribeiro PA, Xavier R, Santos AM, Hawkins SJ (2009) Reproductive cycles of four species of *Patella* (Mollusca: Gastropoda) on the northern and central Portuguese coast. *Journal of the Marine Biological Association of the UK* 89: 1215-1221.

Roberts RD, Nicholson CM (1997) Variable response from abalone larvae (*Haliotis iris, H. virginea*) to a range of settlement cues. *Molluscan Research* 18: 131-141.

Scheltema RS (1986) On dispersal and planktonic larvae of benthic invertebrates: an eclectic overview and summary of problems. *Bulletin of Marine Science* 39: 290-322.

Shanks AL (1998) Apparent oceanographic triggers to the spawning of the limpet *Lottia digitalis* (Rathke). *Journal of Experimental Marine Biology and Ecology* 222: 31-41.

Shanks AL, McCulloch A, Miller J (2003) Topographically generated fronts, very nearshore oceanography and the distribution of larval invertebrates and holoplankters. *Journal of Plankton Research* 25: 1251-1277.

Silva A, Brotas V, Valente A, Sá C, Diniz T, Patarra RF, Álvaro NV, Neto AI (2013) Coccolithophore species as indicators of surface oceanographic conditions in the vicinity of Azores islands. *Estuarine, Coastal and Shelf Science* 118: 50-59.

Strathmann RR, Hughes TP, Kuris AM, Lindeman KC, Morgan SG, Pandolfi JM, Warner RR (2002) Evolution of local recruitment and its consequences for marine populations. *Bulletin of Marine Science* 70: 377-396.

Swearer SE, Shima JS, Hellberg ME, Thorrold SR, Jones GP, Robertson DR, Morgan SG, Selkoe KA, Ruiz GM, Warner RR (2002) Evidence of self-recruitment in demersal marine populations. *Bulletin of Marine Science* 70: 251-271.

Tatsumi M, Wright JT (2016) Understory algae and low light reduce recruitment of the habitat-forming kelp *Ecklonia radiate*. *Marine Ecology Progress Series* 552 131-143.

Teske PR, Sandoval-Castillo J, van Sebille E, Waters J, Beheregaray LB (2016) Oceanography promotes self-recruitment in a planktonic larval disperser. *Scientific Reports* 6: 34205.

Thompson GB (1979) Distribution and population dynamics of the limpet *Patella aspera* (Lamarck) in Bantry Bay. *Journal of Experimental Marine Biology and Ecology* 40: 115-135.

Thompson RC, Norton TA, Hawkins SJ (1998) The influence of epilithic microbial films on the settlement of *Semibalanus balanoides* cyprids - a comparison between laboratory and field experiments. *Hydrobiologia* 375: 203-216.

Underwood AJ (1997) *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge University Press, Cambridge.

Underwood AJ (2004) Landing on one's foot: small-scale topographic features of habitat and the dispersion of juvenile intertidal gastropods. *Marine Ecology Progress Series* 268: 173-182.

Underwood AJ, Denley EJ, Moran MJ (1983) Experimental analyses of the structure and dynamics of mid-shore rocky intertidal communities in New South Wales. *Oecologia* 56: 202-219.

Underwood AJ, Fairweather PG (1989) Supply-side ecology and benthic marine assemblages. *Trends in Ecology and Evolution* 4: 16-20.

Vale M (2016) *Influence of climate change and other impacts on rocky intertidal communities of the Azores.* PhD thesis, University of Southampton.

SUPPLEMENTARY MATERIAL

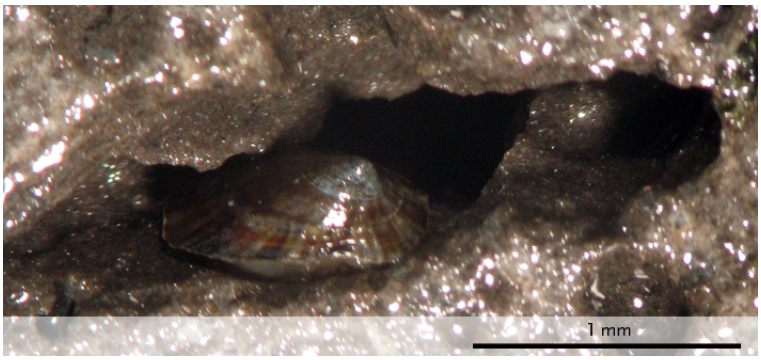


Figure S1. An early recruit of *Patella candei* in a basaltic plate.

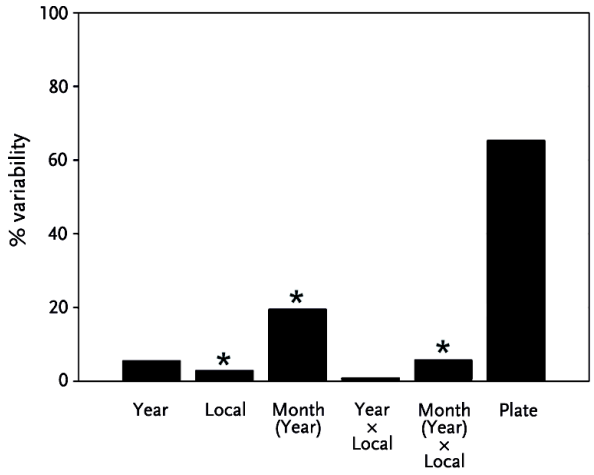


Figure S2. Temporal and spatial components of variability for *Patella candei* recruitment during 2014 and 2015. Significance: * P < 0.05 (see Table S1 for ANOVA terms).

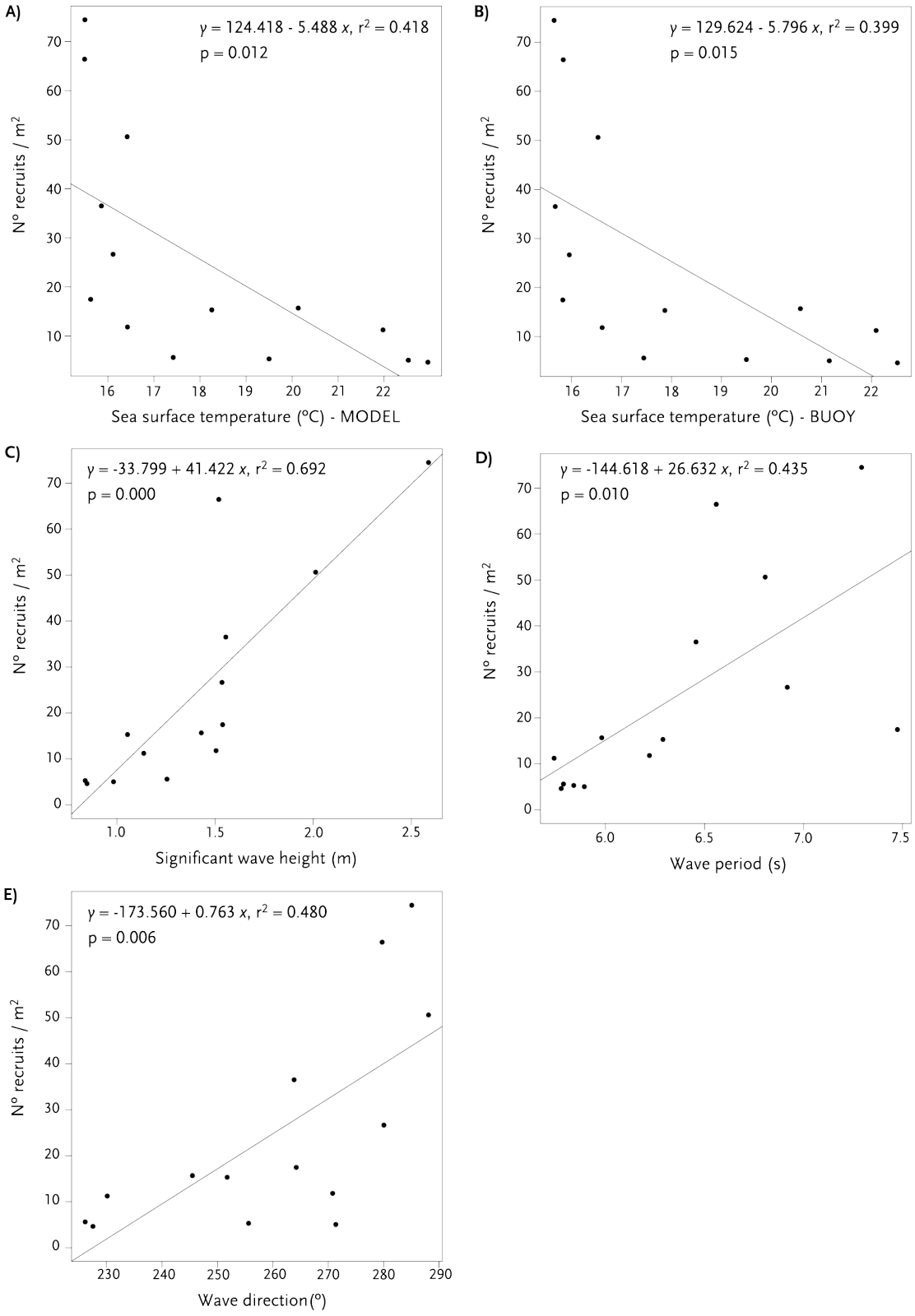


Figure S3. Regression analyses between the total mean number of *Patella candei* recruits and the environmental variables A) sea surface temperature - SST_{MODEL}; B) sea surface temperature - SST_{BUOY}, C) significant wave height (H_S), D) wave period (T_Z) and E) wave direction (W_{DIR}).

Table S1. A three-way ANOVA examining temporal and spatial differences in *Patella candei* recruitment. Analysis performed on untransformed data and the significance level set to α = 0.01.

	df	MS	F
YEAR	1	8.12E+04	3.39
LOCATION	3	1.74E+04	7.67***
MONTH (YEAR)	22	1.98E+04	8.69***
YEAR × LOCATION	3	4.83E+03	2.13
MONTH (YEAR) × LOCATION	66	2.27E+03	2.34***
Residual	1344	9.73E+02	
Total	1439		

*** $P < 0.001$

Table S2. A three-way ANOVA examining temporal and spatial differences in *Patella candei* peak recruitment (from January to April on 2014 and 2015).

	df	MS	F
YEAR	1	303.12	11.921**
LOCATION	3	6.98E+01	19.49***
MONTH (YEAR)	6	1.34E+01	3.73*
YEAR × LOCATION	3	1.24E+01	3.45*
MONTH (YEAR) × LOCATION	18	3.58E+00	1.19
Residual	448	3.02E+00	
Total	479		

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table S3. A two-way ANOVA examining temporal differences in environmental variables obtained from the moored buoy.

Sea surface temperature - SST_{BUOY}	df	MS	F
YEAR	1	2.45E+01	4.890
MONTH (YEAR)	12	5.02E+00	No test
Total	13		
Significant wave height - H_S			
YEAR	1	8.55E-01	5.076*
MONTH (YEAR)	12	1.68E-01	No test
Total	13		
Wave period - T_Z			
YEAR	1	8.02E-01	2.693
MONTH (YEAR)	12	2.98E-01	No test
Total	13		
Wave direction - W_{DIR}			
YEAR	1	1.46E+03	3.973
MONTH (YEAR)	12	3.68E+02	No test
Total	13		

* $P < 0.05$

CHAPTER 7

Larval development of the limpet *Patella candei* at varying water temperatures: implications under global warming

ABSTRACT

Temperature can have profound effects on larval growth rates, which in turn impact pelagic larval duration of many free-spawning marine invertebrates. Under predictive scenarios of global warming it is important to understand the likely changes in distribution, dispersal ability and population structure of marine species, especially those that are commercially exploited. Here, the effect of water temperature on larval development of *Patella candei* (d'Orbigny 1840) from Azores (NE Atlantic) was examined under controlled laboratory conditions. Three temperatures that are usually experienced in the waters surrounding the archipelago throughout the year were used (14ºC, 18ºC and 22ºC). Both the duration of larval development and larval survivorship were significantly affected by temperature. Larval development was faster at increasing temperatures and cumulative survivorship decreased; about 25% of larvae at 22ºC survived to the end of the experiment, a 2-fold decrease from the average survivorship of ~ 50% at 14ºC. As sea surface temperatures continue to rise, embryos and larvae of *P. candei* will be subject to a deleterious effect, probably reducing the dispersal capacity and the recovery potential of exploited populations of *P. candei* throughout the region. Experimental trials on larval development provide valuable information that may be used to refine current and future transport models for geographically-isolated, exploited limpets such as *P. candei* under changing environmental conditions, helping to adjust conservation and resource-management actions aimed at protecting such important and endemic resources.

KEYWORDS: climate change, ocean warming, dispersal, larvae development, free-spawning invertebrates

Manuscript in preparation as:

Faria J, Martins GM, Hawkins SJ, Neto AI, Ribeiro PA. *In prep*. Larval development of the limpet *Patella candei* at varying water temperatures: implications under global warming.

1. Introduction

Wide-ranging evidence shows that ocean warming has been taking place for the last decades (IPCC 2014) and current trends emphasize the need for a deeper understanding of the mechanisms of effects on global marine species and communities. The energetic metabolism of living organisms is largely dependent on temperature (Brown *et al.* 2004), being affected by both unfavourably high and unfavourably low temperatures. Thus ocean warming is likely to affect the development, growth and metabolic traits of individuals, thereby, influencing the distribution, abundance, population dynamics and reproductive timing of species (see reviews in Helmuth *et al.* 2006; Byrne 2011). This will inevitably lead to changes in the functioning of many marine ecosystems, with some species probably facing extinction while others will thrive (see Hawkins *et al.* 2009; Hoegh-Guldberg and Bruno 2010). In particular, early life-stages of free-spawning marine invertebrates can be especially vulnerable to climatic fluctuations and strongly influencing subsequent life-history stages (Gosselin and Qian 1996, 1997). In fact, larvae represent a physiological bottleneck, as they are generally more sensitive to thermal, nutritional and other environmental stresses than their conspecific adults (Anger 2001). For instance, numerous studies on the pace of developmental constraints, swimming performance, physiology and planktonic larval duration (PLD) have showed that temperature acts as a major environmental factor controlling invertebrate development (e.g. Anger *et al.* 2003; Bassim and Sammarco 2003; Green and Fisher 2004; Brennand *et al.* 2010; Arnberg *et al.* 2013; David and Simon 2014). Although the response of experimentally-reared marine invertebrate embryos and larvae under distinct temperature regimes varied between species and studies (Negri *et al.* 2007; Byrne 2012), increasing temperature has a generalised pervasive stimulatory effect on metabolism until lethal levels are reached (see Supporting information in O'Connor *et al.* 2007). This has important consequences for a range of ecological attributes including larval dispersal, population connectivity, local adaptation, and speciation in marine biota. Given that the duration of the larval period is known to influence larval dispersal distance and survival, which in turn are positively correlated with population connectivity (Bohonak 1999), genetic structure is likely to be higher in warmer waters, or can increase under the predicted scenario of ocean warming. In fact, a positive correlation between latitude and connectivity among species has been previously detected in polyplacophoran molluscs, with species at lower latitudes generally having more isolated (hence structured) populations (e.g. Kelly and Eernisse 2007). This pattern is probably driven by faster larval development in warmer waters reducing the duration of the dispersive pelagic stage and consequently the connectivity among populations, by limiting the dispersal potential of the species. Although such observations may not be extended to all living taxa, as many other factors can be involved, understanding the way temperature influences the development of early live-stages can provide valuable information to more realistic biophysical models of dispersal of free-spawning marine invertebrates (e.g. Paris *et al.* 2013). The ability to assess the degree of demographic connectivity over ecological and evolutionary time frames, through the identification of putative barriers to dispersal and range limitations, will definitely aid the re-shaping of present-day marine reserves, while fostering better management strategies under expected scenarios of climatic changes.

The endemic limpet *Patella candei* (d'Orbigny 1840) is distributed across the Macaronesia archipelagos of Azores, Madeira and Canaries. For discussion of taxonomic status within each archipelago see Faria *et al.* (2017). Although there are many references in the literature to studies on the reproduction of intertidal gastropods, very little is known about the development, morphology and environmental requirements of *P. candei* larvae. Most available information is derived from other *Patella* congeners for which the development and reproductive cycle have been described (i.e. Orton *et al.* 1956; Bowman and Lewis 1986; Espinosa *et al.* 2002; McCarthy *et al.* 2008; Ribeiro 2008; Ribeiro *et al.* 2009; Prusina *et al.* 2014). In Azores, *P. candei* is known to be mostly a winter-breeder, although recruits can be found throughout the year (see Chapter 6). Although simultaneous hermaphroditic individuals have been found before and likely associated to a one-time aberration (Cunha *et al.* 2007), sexes in *P. candei* are separated and fertilization is external. When reproductively active, mature male and female gametes are released directly into the water column, and upon fertilization, larvae undertake a series of development stages until they settle and recruit in suitable substrata. Because ocean warming is likely to influence larval duration, dispersal and survival, this study aims to investigate the influence of temperature on larval development of *P. candei*. This will improve our ability to predict *P. candei* larval dynamics throughout its distributional area under projected scenarios of ocean warming, and may help to re-design protective management measures.

2. Methods

2.1. Collection and treatment of gametes

Experimental treatments were carried out in wintertime, from November 2015 to March 2016, to ensure that sampling could take place during *P. candei* reproductive peak in Azores (see Chapter 6 and Vale 2016), thereby increasing the chances of collecting individuals with fully mature gonads. A total of 50 individuals of *P. candei* (shell length > 30 mm) were collected from a rocky shore platform in São Miguel island, Azores (37º443'9.33''N - 25º38'20.94''W). Individuals were kept alive and immediately transported to the laboratory in a cool box for processing. Limpets were then dissected to determine their sex through gonad colouration and the gonads were staged following the semi-quantitative scale of Orton *et al.* 1956 (Fig. 1). Only individuals showing a fully developed gonad (stage V) were selected for in-vitro fertilization trials. For sperm stock, male gonads were carefully punctured and a few µl of undiluted sperm were added to 500 ml of filtered and UV sterilised seawater (FSW). The solution was filtered through a 300 µm mesh to remove gonad debris and then allowed to rest for 30 minutes (sperm is immobile and becomes active when in contact with seawater for a certain period of time). Sperm motility and concentration were checked in a Neubauer chamber (chamber depth 0.1 mm) under an optical microscope. The treatment of individual limpets and larvae was performed under room temperature (~ 19ºC) and sterilized conditions were maintained throughout the entire procedure. Sperm stock solution was used within the hour. The same ripening/ filtering approach was performed on stage V female gonads. Because oocyte/egg alkalinisation is known to improve fertilization rates (see Hodgson *et al.* 2007; Pérez *et al.* 2016), clean oocytes were placed for 2 hours in 500 ml FSW with pH = 9.0 (adjusted with NH_4OH) before fertilization trials.

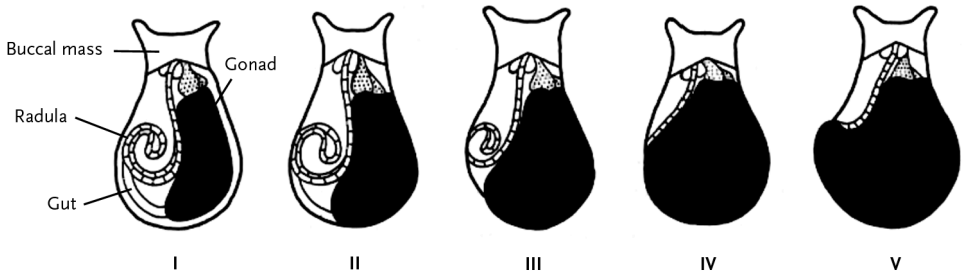


Figure 1. Diagrammatic representation of gonad development stages in *Patella* (adapted from Orton *et al*. 1956). This semi-quantitative scale scores gonad development, ranging between empty neuters (sex and gonad indistinguishable) to fully developed gonads (stage V). In *P. candei*, female gonads are dark brown, whereas in males, the gonad is yellow to orange.

2.2. Experimental procedure

Fertilization experiments were performed under controlled conditions of light and temperature using three environmental test chambers (SANYO). Given the sea surface temperature range in the Azores throughout the year (roughly ranging from 14ºC to 22ºC), three constant water temperatures were selected for fertilization trials: 14, 18 and 22ºC. In each case, a gentle airflow system and a 12:12 h day/night cycle was applied for a total of three rearing replicates. In each replicate, batches of cleaned eggs (pre-treated in pH 9 FSW) were placed in 100 ml FSW glass beakers, for a final concentration of ~ 70 eggs ml^{-1}. To achieve higher fertilization rates (see Hodgson *et al.* 2007), a pre-adjusted sperm stock volume was added to each beaker so that a final concentration of ~ 10^6 sperm ml^{-1} was achieved. The average stage of development in each replicate culture was determined by visual examination of larvae under a microscope of three 1ml subsamples taken at pre-defined times (T: 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h). Before sampling, cultures were gently stirred to homogenise the distribution of larvae in the water. Samples were placed in 1.5 ml tubes and fixed with one drop of 5% formalin. In all samples, the numbers of unfertilized eggs, embryo on initial process of cell division (cleavage) with 2-4 blastomeres, morula, swimming trochophore and pre- and post-torsional veliger were recorded. After 45 h upon fertilization, larvae were fed periodically (24 h intervals) with *Phaedactylum tricornutum* (preliminary trials showed that *P. candei* larvae are planktotrophic). Moreover, after each sampling event, a 64 µm mesh filter was carefully used to decant larvae onto 100 ml of freshly made FSW. The FSW was previously stored at each experimental temperature, so as to avoid temperature oscillations during water changes.

2.3. Statistical analyses

For each sampling time, chi-square tests were used to evaluate the relationship between stages of development (STAGE) and temperature (TEMP). Moreover, differences between treatments were examined using a 2-way permutational ANOVA with the following factors: TEMP (3 levels; fixed) and STAGE (6 levels; fixed). All tests were done using PERMANOVA+ based on Euclidean distances and using 999 permutations (Anderson *et al.* 2008). Prior to analyses, data were checked for homogeneity of variance and transformations were applied when needed (Underwood 1997). Analyses were

performed on untransformed data when attempts to stabilize variances through transformation were unsuccessful; in such cases, a more conservative significance level ($\alpha = 0.01$) was used when testing for differences (Underwood 1997). Pairwise tests were used to resolve significant differences within stages. Survival rates were determined for all temperatures at every pre-defined time.

3. Results

This study showed, for the first time, that *P. candei* larvae are planktotrophic as they were observed feeding on diatoms. Experimental trials were therefore conducted on feeding larvae. As expected, the sequence of development stages observed in this study was similar to others related limpet species (Dodd 1957; Wanninger *et al.* 1999; Espinosa *et al.* 2002; Ribeiro 2008). Upon fertilization, embryos undergo a series of cleavages before they develop into the so-called trochophore larvae typical for molluscs. They are pear-shaped and girdled by a ring of cilia that enables them to swim actively, both horizontally and vertically, with steady swimming often alternated with abrupt bursts of speed. Before they can metamorphose into the adult form, the trochophore develops into a pre and post-torsional veliger. The trochophore-veliger transition involves torsion, which is the clockwise turn of the visceral portion relative to the head-foot portion (Ghiselin 1966), the development of embryonic shell and formation of the operculum. Upon completion of torsion, veligers are able to withdraw into their shells, exhibit cephalic tentacles, both eyespots are clearly noticeable and the foot is developed. The latter will be used to adhere to the substrate during the settlement of competent larvae. Unfortunately, complete mortality occurred at all temperatures after T = 72 h within the post-veliger stage, and final metamorphoses were not observed.

With the exception of T = 6 h, chi-square tests revealed that, the observed proportion of larval development stages at each sampling time were not independent of temperature (Table S1). Moreover, significant interaction terms were detected in most ANOVA analyses (except in T= 6 h and T = 12 h; Fig. 2 and Fig. S1). Generally, the proportion of early stages decreased with time and this was more evident at increasing temperatures. Later stages (i.e. trochophore and veligers) were also found sooner at higher temperatures (Fig. 2). For instance, larvae at 14ºC took more than twice the time to reach the veliger state then at 22ºC (median development time was estimated as the time required by at least 50% of the observed larvae to reach a given stage).

In addition to an acceleration in development, a greater cumulative percentage of embryos and larvae exhibited abnormal development at higher temperatures. Although no significant differences were detected in instantaneous survival rates across temperatures (identical slopes), the cumulative survivorship decreased in time and was consistently lower at higher temperatures (Fig. 3; Table 1). For example, about 25% of larvae survived until the end of experiment (72 h) at 22ºC, about half the average survival (~ 50%) recorded at 14ºC. Differences found among temperatures were mainly determined at initial stages of development (Fig. 3).

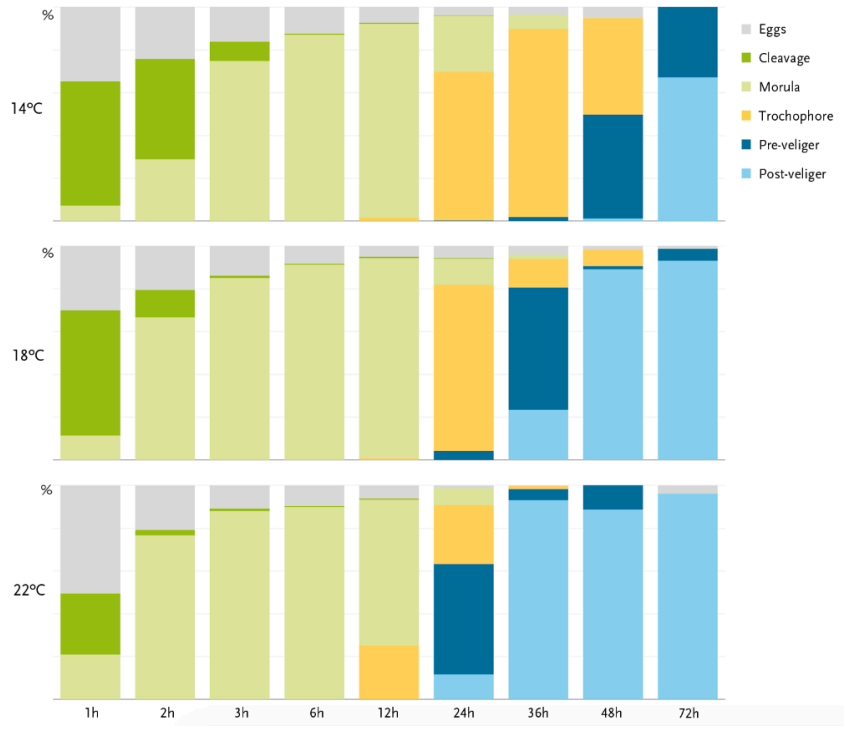


Figure 2. Proportion of larval development stages of *Patella candei* reared at three constant temperatures (14, 18 and 22ºC) during 72 h.

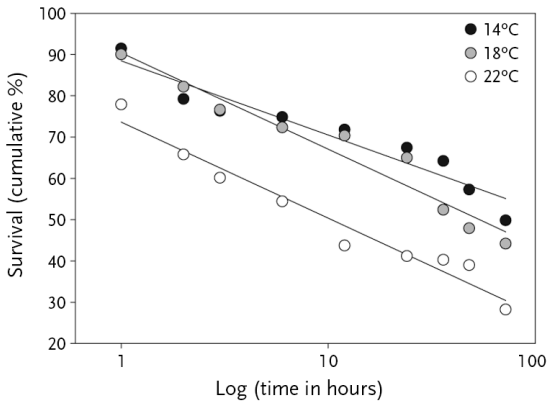


Figure 3. Survival (cumulative %) of *P. candei* larvae reared at three constant temperatures (14, 18 and 22ºC) during 72 h.

Table 1. A two-way PERMANOVA examining differences in larvae survivorship among temperature treatments. Sampling time was used as the covariate.

	df	MS	F
TIME	1	4787.6	311.64**
TEMPERATURE	2	1047.7	68.2**
TIME × TEMPERATURE	2	32.341	2.1052
Residual	21	15.362	
Total	26		
Pairwise comparisons			
14ºC = 18ºC; 14ºC ≠ 22ºC; 18ºC ≠ 22ºC			

** $P < 0.01$

4. Discussion

This study tested the response of *P. candei* embryos and larvae to temperature conditions that are usually experienced in the waters surrounding the archipelago of Azores throughout the year (Amorim *et al.* 2017). Being winter brooders, *P. candei* larvae in Azores are expected to perform better and attain higher fitness at colder temperatures. In fact, results showed that development was accelerated but the percentage of developmental abnormalities increased at higher temperatures, negatively affecting the cumulative survivorship of larvae. Consequently, as sea surface temperatures continue to rise, embryos and larvae of *P. candei* will be subject to a deleterious effect, likely reducing the time window each year for successful reproduction and larval development. Thus recruitment and the recovery potential of exploited populations of *P. candei* will be diminished throughout its distribution area. Relatively small perturbations in recruitment can translate to large alterations of adult populations (Uthicke *et al.* 2009). If these are accompanied by high levels of exploitation, the resilience and survival of the species can be greatly threatened. Therefore, understanding the exact capacity of a given species to disperse under distinct environmental conditions is especially important in fisheries planning and implementation of conservation measures. Unfortunately, due to poor rearing conditions or intrinsic limitations while culturing invertebrate larvae in vitro (e.g. proliferation of pathogens; excessive larval manipulation during water changes; larval density constraints), the final metamorphosis stage was never detected, and the full extent of the PLD in *P. candei* could not be determined. However, increasing the larval development rate in *P. candei* under increasing temperatures, will likely reduce the full potential of dispersal and population connectivity, with individuals recruiting more locally near their source adult populations, hence increasing structure among populations. At the very least, results indicate that the PLD of *P. candei* is over three days; in all experimental conditions, actively swimming larvae were observed 3 days (72 h) after fertilization and before full larvae mortality occurred. This estimate, however, can be further reduced. For instance, Ribeiro (2008) showed that patellid larvae accelerate their development and reach metamorphosis faster when in the presence of their preferred settling substrata (i.e. crustose red algae-covered stones) supporting other earlier studies showing that metamorphosis can only be achieved if suitable settlement inducers are present (Smith 1935; Dodd 1957).

While the relatively cold adapted and planktotrophic nature of *P. candei* larvae in Azores may suggest a broader geographic distribution of the species throughout the NE Atlantic region, current populations are restricted to the archipelagos of Azores, Madeira and Canaries, and even these exhibit striking dissimilarities among them (Faria *et al.* 2017). As so, and aside from evolutionary and phylogeographic considerations, the suggestion that the PLD of *P. candei* might be shorter than expected and the occurrence of oceanographic features such as eddies, currents, and meanders are likely to be the most prominent factors limiting the connectivity capacity between populations of *P. candei* among all archipelagos and elsewhere. Particular adaptations to each archipelago environmental conditions can also act to determine restrictions in population connectivity and individual dispersal. The adaptability of *P. candei* to regional climate does not imply strict dependencies for the species throughout its area of distribution. The temperature dependence of

larval development within a species may vary with latitude due to local adaptations of geographically distant populations, a phenomenon that has been previously reported (Drent 2002). Due to, and also in support of the isolated nature of *P. candei* populations (see Faria *et al.* 2017), future research should search for inter-specific variation in PLD at any given temperature across the entire distribution of *P. candei sensu lato* throughout the archipelagos of Azores, Madeira and Canaries, and examine if an evidence for variation can be explained by specific metabolic adaptations to particular environmental conditions. Indeed, multi-population comparisons are urgently needed to better predict pending ecosystem changes.

The observed pattern of sensitivity of *P. candei* to higher temperatures also explains the reproductive timing of the species in Azores. As previously described (see Chapter 6), the yearly recruitment in *P. candei* is known to peak between January and March, which implies the presence of larvae in open waters a few days or weeks before, during the North hemisphere winter season. With monthly temperatures ranging between 14 - 16ºC (Amorim *et al.* 2017), this time of the year seems to provide the necessary and optimal temperature conditions for larvae to develop and survive. Moreover, although growth was not measured in this study, *P. candei* larvae at colder temperatures may be less developed at a given size, but are more likely to settle at smaller sizes than larvae reared in warmer conditions (e.g. Laurel *et al.* 2014). Yet, if larval rearing could have been performed under colder temperatures, the relationship between temperature and survivorship would likely follow a dome-shaped curve, as lower temperatures outside the species optimal range would also reduce the survival of individuals (Munday *et al.* 2009).

The effect of temperature on the developmental rate of marine invertebrates has long been recognized with many studies showing that increasing temperatures accelerate larval development and provide a deleterious effect to larval survivorship (e.g. Anil *et al.* 2001; Thiyagarajan *et al.* 2003; Randall and Szmant 2009; Bashevkin and Pechenik 2015). In some cases, however, warmer temperatures may provide favourable conditions to the early life stages of free-spawning marine invertebrates, as increasing growth rates will make larvae larger and less vulnerable to size-specific predation (Bashevkin and Pechenik 2015; Miller *et al.* 1988). Nevertheless, the PLD of a given species will be inevitably affected if temperatures are likely to increase. For instance, the relationship between PLD and temperature derived by O'Connor *et al.* (2007), based on 69 species of invertebrates and fish of mostly temperate distribution, shows that an increase of 1°C from 14 to 15°C should decrease PLD by ~ 10% and a 3°C temperature increase from 14 to 17°C should decrease PLD by ~ 25.5%. Although temperature may be the single most important factor determining development and growth rates of early life stages in marine invertebrates, the capacity of adult populations to be replenished by new individuals is dependent on a vast number of factors (e.g. larval supply; food availability) that can act collectively in determining the eventual distribution and abundance of a species (Cowen and Sponaugle 2009). In order to assess the potential outcomes for *P. candei* in a changing ocean, future studies should include multiple stressors experiments on larval development, tolerance and survivorship, and assess the interactive effects of factors such as temperature, salinity and food availability on larval success. These should not only focus on various life-history stages, but also different populations of a species (Helmuth *et al.* 2006). This study emphasizes the importance of

including larval development information in models attempting to predict the effects of climate change on species distributions. Such data may be used to refine current and future transport models for exploited limpets such as *P. candei* under changing environmental conditions in the North Atlantic archipelagic region, helping in adjusting conservation and resource-management actions aimed at protecting such important and endemic resource.

Acknowledgments

We thank Zaira Nogueira for field and laboratory assistance during the course of rearing experiences. JF was funded by a PhD grant M3.1.2/ F/021/2011 by the Regional Government of the Azores.

References

Amorim P, Perán AD, Pham CK, Juliano M, Cardigos F, Tempera F, Morato T (2017) Overview of the ocean climatology and its variability in the Azores region of the North Atlantic including environmental characteristics at the seabed. *Frontiers in Marine Science* 4:56.

Anderson MJ, Gorley RN, Clarke KR (2008) *PERMANOVA for PRIMER: guide to software and statistical methods*. PRIMER-E Ltd., Plymouth, United Kingdom.

Anger K (2001) Contributions of larval biology to crustacean research: A review. *Invertebrate Reproduction & Development* 49: 175-205.

Anger K, Thatje S, Lovrich G, Calcagno J (2003) Larval and early juvenile development of *Paralomis granulosa* reared at different temperatures: tolerance of cold and food limitation in a lithodid crab from high latitudes. *Marine Ecology Progress Series* 253: 243-251.

Anil AC, Desai D, Khandeparker L (2001) Larval development and metamorphosis in *Balanus amphitrite* (Cirripedia: Thoracica): significance of food concentration, temperature and nucleic acids. *Journal of Experimental Marine Biology and Ecology* 263: 125-141.

Arnberg M, Calosi P, Spicer J, Tandberg AHS, Nilsen M, Westerlund S, Bechmann RK (2013) Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (*Pandalus borealis*) larvae. *Marine Biology* 160: 2037-2048.

Bashevkin SM, Pechenik JA (2015) The interactive influence of temperature and salinity on larval and juvenile growth in the gastropod *Crepidula fornicata* (L.). *Journal of Experimental Marine Biology and Ecology* 470: 78-91.

Bassim K, Sammarco P (2003) Effects of temperature and ammonium on larval development and survivorship in a scleractinian coral (*Diploria strigosa*). *Marine Biology* 142: 241-252.

Bohonak AJ (1999) Dispersal, gene flow, and population structure. *The Quarterly Review in Biology* 74: 21-45.

Bowman RS, Lewis JR (1986) Geographical variation in the breeding cycles and recruitment of *Patella* spp. *Hydrobiologia* 142: 41-56.

Brennand SH, Soars N, Dworjanyn SA, Davis AR, Byrne M (2010) Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin *Tripneustes gratilla*. *PlosOne* 5: e11372.

Brown JH, Gillooly JF, Allen AP, Savage VM, West GB (2004) Toward a metabolic theory of ecology. *Ecology* 85: 1771-1789.

Byrne M (2011) Impact of ocean warming and ocean acidification on marine in vertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology* 49: 1-42.

Byrne M (2012) Global change ecotoxicology: identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. *Marine Environmental Research* 76: 3-15.

Cowen RK, Sponaugle S (2009) Larval dispersal and marine population connectivity. *Annual Review of Marine Science* 1: 443-466.

Cunha L, Martins GM, Amaral A, Rodrigues A (2007) A case of simultaneous hermaphroditism in the Azorean endemic limpet *Patella candei gomesii* (Mollusca: Patellogastropoda), a gonochoristic species. *Invertebrate Reproduction & Development* 50: 203-205.

David AA, Simon CA (2014) The effect of temperature on larval development of two non-indigenous poecilogonous polychaetes (Annelida: Spionidae) with implications for life history theory, establishment and range expansion. *Journal of Experimental Marine Biology and Ecology* 461: 20-30.

Dodd JM (1957) Artificial fertilisation, larval development and metamorphosis in *Patella vulgata* L. and *Patella caerulea* L. *Pubblicazioni della Stazione Zoologica di Napoli* 29: 172-185.

Drent J (2002) Temperature responses in larvae of *Macoma balthica* from a northerly and southerly population of the European distribution range. *Journal of Experimental Marine Biology and Ecology* 275: 117-129.

Espinosa F, Rivera-Ingraham GA, García-Gómez JC (2002) Early stages of development in the endangered limpet *Patella ferruginea* Gmelin, 1791 (Gastropoda: Patellidae). *Nautilus* 124: 51-53.

Faria J, Martins GM, Pita A, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017) Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic). *Ecology and Evolution* 7(16): 6125-6140.

Ghiselin MT (1966) The adaptive significance of gastropod torsion. *Evolution* 20: 337-348.

Gosselin LA, Qian PY (1996) Early post-settlement mortality of an intertidal barnacle: A critical period for survival. *Marine Ecology Progress Series* 135: 69-75.

Gosselin LA, Qian PY (1997) Juvenile mortality in benthic marine invertebrates. *Marine Ecology Progress Series* 146: 265-282.

Green BS, Fisher R (2004) Temperature influences swimming speed, growth and larval duration in coral reef fish larvae. *Journal of Experimental Marine Biology and Ecology* 299:115-132.

Hawkins SJ, Sudgen HE, Mieszkowskaa N, Moore PJ, Poloczanska E, Leaper R, Herbert RJH, Genner MJ, Moschella PS, Thompson RC, Jenkins SR, Southward AJ, Burrows MT (2009) Consequences of climate-driven biodiversity changes for ecosystem functioning of North European rocky shores. *Marine Ecology Progress Series* 396: 245-259.

Helmuth B, Mieszkowska N, Moore P, Hawkins SJ (2006) Living on the edge of two changing worlds: Forecasting the responses of rocky intertidal ecosystems to climate change. *Annual Review of Ecology, Evolution, and Systematics* 37: 373-404.

Hodgson AN, Quesne W, Hawkins SJ, Bishop JDD (2007) Factors affecting fertilization success in two species of patellid limpet (Mollusca: Gastropoda) and development of fertilization kinetics models. *Marine Biology* 150: 415-526.

Hoegh-Guldberg O, Bruno JF (2010) The impact on climate change on the world's marine ecosystems. *Nature* 328: 1523-1528.

IPCC (2014) *Climate Change 2014: Synthesis Report*. Contribution of Working Groups I, II and III to the Fifth assessment report of the intergovernmental panel on climate change [Core Writing Team, RK Pachauri, LA Meyer (Eds)]. IPCC, Geneva, Switzerland.

Kelly RP, Eernisse DJ (2007) Southern hospitality: a latitudinal gradient in geneflow in the marine environment. *Evolution* 61: 700-707.

Laurel BJ, Danley C, Haines S (2014) The effects of temperature on growth, development and settlement of northern rock sole larvae (*Lepidopsetta polyxystra*). *Fisheries Oceanography* 23(6): 495-505.

McCarthy M, Woosnam P, Culloty SC (2008) Histological investigation of the reproductive cycles of the limpets *Patella vulgata* and *Patella ulyssiponensis*. *Marine Biology* 153: 871-877.

Miller TJ, Crowder LB, Rice JA, Marschall EA (1988) Larval size and recruitment mechanisms in fishes: toward a conceptual framework. *Canadian Journal of Fisheries and Aquatic Sciences* 45: 1657-1670.

Munday PL, Leis JM, Lough JM, Paris CB, Kingsford MJ, Berumen ML, Lambrechts (2009) Climate change and coral reef connectivity. *Coral Reefs* 28: 379-395.

Negri AP, Marshall PA, Heyward AJ (2007) Differing effects of thermal stress on coral fertilization and early embryogenesis. *Coral Reefs* 26: 759-763.

O'Connor MI, Bruno JF, Gaines SD, Halpern BS, Lester SE, Kinlan BP, Weiss JM (2007) Temperature control of larval dispersal and the implications for marine ecology, evolution and conservation. *Proceedings of the National Academy of Sciences of the USA* 104: 1267-1271.

Orton JH, Southward AJ, Dodd JM (1956) Studies on the biology of limpets. II. The breeding of *Patella vulgata* L. in Britain. *Journal of the Marine Biological Association of the UK* 35: 149-176.

Paris CB, Helgers J, Sebille E, Srinivasan A (2013) Connectivity Modeling System: A probabilistic modeling tool for the multi-scale tracking of biotic and abiotic variability in the ocean. *Environmental Modelling & Software* 42: 47-54.

Pérez S, Fernández N, Ribeiro PA (2016) Standardization of a *Patella* spp. (Mollusca, Gastropoda) embryo-larval bioassay and advantages of its use in marine ecotoxicology. *Ecotoxicology and Environmental Safety* 127: 175-186.

Prusina I, Ezgeta-Balic D, Ljubimir S, Dobroslavic T, Glamuzina B (2014) On the reproduction of the Mediterranean keystone limpet *Patella rustica*: histological overview. *Journal of Experimental Marine Biology and Ecology* 94: 1651-1660.

Randall CJ, Szmant AM (2009) Elevated temperature affects development, survivorship, and settlement of the Elkhorn Coral, *Acropora palmata* (Lamarck 1816). *Biological Bulletin* 217: 269-282.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Ribeiro PA, Xavier R, Santos AM, Hawkins SJ (2009) Reproductive cycles of four species of *Patella* (Mollusca: Gastropoda) on the northern and central Portuguese coast. *Journal of the Marine Biological Association of the UK* 89: 1215-1221.

Smith FGW (1935) The development of *Patella vulgata*. *Philosophical Transactions of the Royal Society B* 225: 95-125.

Thiyagarajan V, Harder T, Qian PY (2003) Combined effects of temperature and salinity on larval development and attachment of the subtidal barnacle *Balanus trigonus* Darwin. *Journal of Experimental Marine Biology and Ecology* 287: 223-236.

Underwood AJ (1997) *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge University Press, Cambridge.

Uthicke S, Schaffelke B, Byrne M (2009) A boom-bust phylum? Ecological and evolutionary consequences of density variations in echinoderms. *Ecological Monographs* 79: 3-24.

Vale M (2016) *Influence of climate change and other impacts on rocky intertidal communities of the Azores.* PhD thesis, University of Southampton.

Wanninger A, Ruthensteiner B, Lobenwein S, Salvenmoser W, Dictus WJAG, Haszprunar G (1999) Development of the musculature in the limpet *Patella* (Mollusca, Patellogastropoda). *Development Genes and Evolution* 209: 226-238.

SUPPLEMENTARY MATERIAL

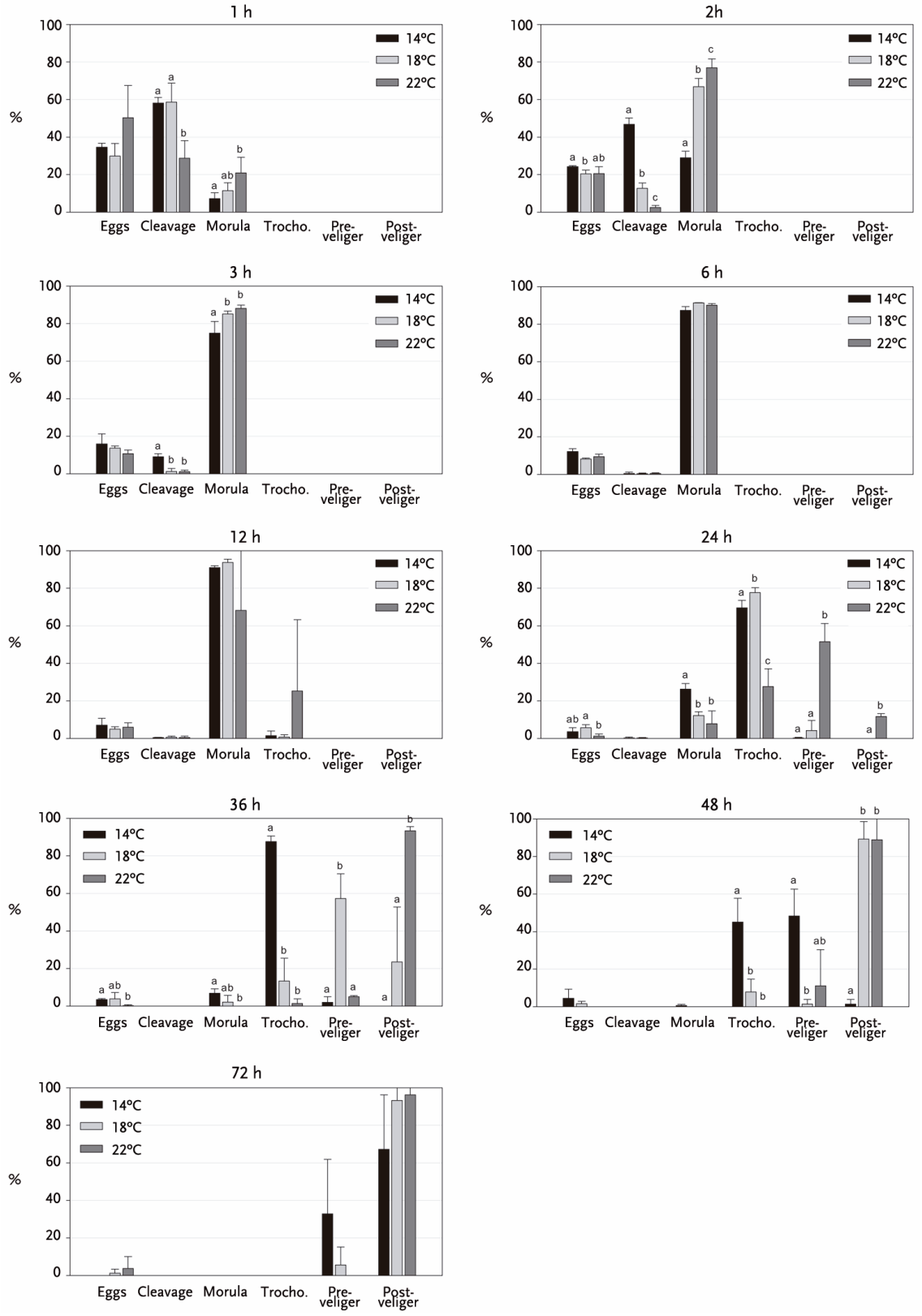


Figure S1. Temporal proportions of each larvae development stage reared at three constant temperatures (14, 18 and 22ºC). Letters indicate non-significant/significant differences in ANOVA pairwise comparisons.

Table S1. Chi-square tests and two-way PERMANOVAs examining differences in the proportion of larval development stages for a given time (T).

T = 1h

Chi-square test						
25.6***						
2-way PERMANOVA	df	F				
TEMP	2	1.6E-13				
STAGE	5	117.9***				
STAGE × TEMP	10	7.8***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.294	0.932	0.245	-	-	-
14 vs. 22	0.189	**0.005**	**0.050**	-	-	-
18 vs. 22	0.130	**0.015**	0.141	-	-	-

T = 2h

Chi-square test						
74.5***						
2-way PERMANOVA	df	F				
	df	F				
TEMP	2	6.4E-13				
STAGE	5	901.4***				
STAGE × TEMP	10	139.5***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	**0.031**	**0.001**	**0.001**	-	-	-
14 vs. 22	0.169	**0.001**	**0.001**	-	-	-
18 vs. 22	0.945	**0.005**	**0.050**	-	-	-

T = 3h

Chi-square test						
13.3**						
2-way PERMANOVA	df	F				
TEMP	2	9.2E-13				
STAGE	5	2091.8***				
STAGE × TEMP	10	9.8***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.511	**0.002**	**0.050**	-	-	-
14 vs. 22	0.198	**0.003**	**0.024**	-	-	-
18 vs. 22	0.102	0.984	0.104	-	-	-

T = 6h

Chi-square test						
0.95						
2-way PERMANOVA	df	F				
TEMP	2	4.1E-01				
STAGE	5	1234.1***				
STAGE × TEMP	10	0.7				
Residual	36					

continue

continued

T = 12h

Chi-square test						
47.9***						
2-way PERMANOVA	df	F				
TEMP	2	5.5E-01				
STAGE	5	63.5***				
STAGE × TEMP	10	1.36				
Residual	36					

T = 24h

Chi-square test						
150.6***						
2-way PERMANOVA	df	F				
TEMP	2	8.2E+00**				
STAGE	5	110.1***				
STAGE × TEMP	10	25.9***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.229	0.880	**0.005**	**0.050**	0.170	-
14 vs. 22	0.137	0.414	**0.045**	**0.016**	**0.001**	**0.001**
18 vs. 22	**0.031**	0.350	0.268	**0.007**	**0.013**	**0.001**

T = 36h

Chi-square test						
350.5***						
2-way PERMANOVA	df	F				
TEMP	2	3.9E+01***				
STAGE	5	1.2E-13				
STAGE × TEMP	10	48.2***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.860	-	0.123	**0.001**	**0.001**	0.257
14 vs. 22	**0.002**	-	**0.008**	**0.001**	**0.155**	**0.001**
18 vs. 22	0.158	-	0.372	0.144	**0.003**	**0.01**

T = 48h

Chi-square test						
218.6***						
2-way PERMANOVA	df	F				
TEMP	2	8.1E-15				
STAGE	5	6.7E+01***				
STAGE × TEMP	10	31.8***				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.356	-	0.147	**0.010**	**0.004**	**0.001**
14 vs. 22	0.176	-	0.155	**0.002**	0.060	**0.001**
18 vs. 22	0.134	-	-	0.127	0.441	0.973

continue

continued

T = 72h

Chi-square test						
58.5***						
2-way PERMANOVA	df	F				
TEMP	2	5.5E-15				
STAGE	5	9.7E+01***				
STAGE × TEMP	10	3.1**				
Residual	36					
Pairwise comparisons (P-values)	EGGS	CLEA	MORU	TROC	VPRE	VPOS
14 vs. 18	0.366	-	-	-	0.180	0.218
14 vs. 22	0.348	-	-	-	0.126	0.172
18 vs. 22	0.544	-	-	-	0.356	0.627

** P < 0.01, *** P < 0.001; Significant P-values in Pairwise comparisons are highlighted in bold; EGGS = unfertilized eggs; CLEA = eggs on initial process of cell division (cleavage); MORU = morula stage; TROC = trochophore; VPRE = pre-torsion veliger; VPOS = post-torsion veliger

CHAPTER 8

Overview and general discussion

Discussion

Summary of thesis findings

The main reasoning behind this PhD lies in the current need for better management practices and conservation strategies for the protection of two commonly exploited limpet species (i.e. *Patella candei* and *P. aspera*) from the Macaronesia archipelagos (NE Atlantic). This thesis had three major objectives that included the analysis of genetic, recruitment and larval development variability; these three aspects are crucial in providing a holistic understanding of the dynamics and resilience to exploitation of these populations.

For the first time, species-specific microsatellite genetic markers for both species were developed. A total of 12 and 17 markers were characterized for *P. candei* and *P. aspera*, respectively [Chapter 2 (Faria *et al.* 2016) and Chapter 3 (Faria *et al.* 2015)]. These were amplified in optimized multiplex reactions and used to investigate species' genetic diversity, population structure and connectivity across Azores, Madeira and Canaries archipelagos [Chapter 4 (Faria *et al.* 2017a) and Chapter 5 (Faria *et al.* 2017b)]. On top of evolutionary and ecological considerations, genetic analyses also provided important information of applied interest for the management of the fishery across the region.

Secondly, a monitoring programme to investigate the spatial and temporal variation in recruitment of *P. candei* was established (Chapter 6). Genetic and recruitment data, along with other aspects of the reproductive cycle of the species, provide vital information for the fine-tuning of conservation actions aimed at fostering the sustainable exploitation of the resource (e.g. seasonal fishing closures). The fine scale of the monitoring programme also allowed exploring the relative importance of environmental conditions (e.g. sea surface temperatures) in driving temporal and spatial variation in recruitment and identifying the relevant scale at which physical processes are coupled with larval recruitment.

Thirdly, an experimental rearing procedure was established to investigate the influence of temperature on larval development of *P. candei* (Chapter 7). Understanding the role of temperature on larval development is not only important to foresee any developmental changes expected under current predictions of future climate change, but also to produce high-resolution bio-physical models that are able to simulate and model larval transport in its natural environment, following its life-history traits, assessing at the same time the role of transport in regulating population connectivity, and the role of different biological and physical factors on dispersal.

The main findings can be briefly summarized as follows:

- Twelve microsatellite markers were developed for *Patella candei*;

- Seventeen microsatellite markers were developed for *Patella aspera*;
- Low genetic diversity was detected for both species across the Macaronesian archipelagos of Azores, Madeira and Canaries;
- Morphological differentiation among archipelagos was detected in *P. candei*;
- Populations of *P. candei* from each archipelago are genetically differentiated and/or ecologically isolated;
- Populations of *P. aspera* from Azores are genetically distinct from those of Madeira and Canaries;
- There was no evidence for population genetic structure within archipelago (i.e. among islands in Azores) for any of the species studied;
- High inbreeding in *P. aspera* is likely a consequence of human harvesting;
- A marked peak in recruitment of *P. candei* occurred during winter/spring months and was found to be positively associated with significant wave height;
- Increasing temperature significantly shortens the time required for larval development in *P. candei*, but negatively affects its survivorship.

Genetic diversity, population structure and connectivity of limpets in Macaronesia

The observed levels of genetic variability of *P. candei* and *P. aspera* populations throughout Macaronesia were relatively low when compared to other patellid species inhabiting the mainland (e.g Perez *et al.* 2007; Ribeiro *et al.* 2010). Whether this is a consequence of the isolated nature of populations inhabiting remote oceanic islands and/or the influence of over-harvesting, the relatively impoverished genetic diversity observed in both species may render them more susceptible to environmental changes. On the one hand, it is not uncommon that small, isolated populations and/or populations at the range edges, exhibit relatively lower levels of genetic diversity compared to more connected populations, or those living in the middle of the species range, both on neutral or non-neutral markers, and that insular populations have less genetic variation than their mainland counterparts (Frankham 1997). If dispersal between mainland and insular populations is completely restricted, then genetic variability in such populations can only be regenerated via mutation, with genetic drift and inbreeding playing a dominant role on reducing such variability (Frankham 1996). Moreover, under limited availability and variability of suitable niches, isolated populations will often exhibit very specific and localised adaptive traits (for review on local adaptations see Sanford and Kelly 2011), and therefore their potential to adapt to any environmental changes may be narrow (or beneficial, when considering local adaptation as a direct evidence that species are capable of evolving in the face of environmental change; see overview in Conover 1998). In such cases, the 'raw material', hence genetic variation, for evolutionary change is restricted and isolated populations become more susceptible to demographic and environmental stochasticity processes and changes (e.g. Eldridge *et*

al. 1999). Considering the Macaronesia region and given the environmental particularities of each archipelago, patellid populations are likely to have preserved distinct adaptive traits and subsets of alleles; if individuals could migrate freely among archipelagos, one would have expected more genetically diverse populations. On the other hand, the anthropogenic pressure on populations, through the constant removal of individuals, may negatively affect the genetic pool of limpets, leading to putative deleterious effects on the species adaptive potential and survivorship (see Walsh *et al.* 2006; Allendorf and Hard 2009). This is further enhanced by the isolated nature of populations. For instance, the induced genetic changes created by the size-biased removal of individuals in *P. aspera* populations, and given the species specific life-history traits, provides a good example of how humans, via harvesting, can influence the genetic composition of populations (Faria *et al.* 2017b; Smith *et al.* 1991; Hauser *et al.* 2002; Pinsky and Palumbi 2014).

Two major aspects were revealed following population structure analyses of *P. candei* and *P. aspera* samples across the Macaronesia region: firstly, populations from Azores are genetically and probably ecologically isolated from those of Madeira and the Canaries; secondly, unrestricted migratory movements associated to relaxed oceanographic barriers and adequate larval dispersal potential are likely responsible for maintaining genetic homogeneity across patellid populations throughout the archipelago of Azores. Hence, patellid larvae are able to develop and disperse among islands, probably contributing for a metapopulation status of limpets in this remote archipelago. However, the presence of genetic homogeneity among populations may not be fully indicative that the populations are significantly demographically connected. By definition, demographic connectivity considers that the level of exchange must be sufficient to impact the demographic rates of the local population(s) (Lowe and Allendorf 2010); it is several orders of magnitude larger than the level of exchange required for the maintenance of genetic homogeneity among subpopulations. Whereas relatively few migrants (or lots if populations are very large) among subpopulations can lead to genetic homogeneity (e.g. Díaz-Viloria *et al.* 2009; Goldstien *et al.* 2009; Hellberg 2009), the proportion of migrants necessary to lead to a significant demographic connectivity among populations, although arbitrary, needs to be higher and occur frequently (i.e. in most years, Hawkins *et al.* 2016). Such demographic connectivity of meta-populations is strongly dependent on conditions such as larval dispersal, habitat quality, adult density and mortality on recipient populations. Just because two populations are genetically undifferentiated does not necessarily mean they are connected by consistent gene flow events (e.g. are *P. aspera* populations in Madeira and Canaries truly connected?). This level of uncertainty has strong implications for conservation purposes, and highlights the importance of complementing genetic data with population dynamics and demography studies to understand whether a natural system fits a metapopulation model or not (see Lamy *et al.* 2012).

Despite the relatively short geographic distance between Madeira and Canaries, there was a significant genetic and morphological distinction among *P. candei* populations from these archipelagos. Without any major recognisable oceanographic barriers, one would expect that Selvagens, which stands almost in the middle of these two archipelagos, would work as a stepping-stone habitat, allowing the movement of individuals and facilitating connectivity among archipelagos. Interestingly, unlike that found for *P. candei*, there was no evidence of genetic structuring in

populations of *P. aspera* from Madeira and Canaries. Species-specific life-history traits (e.g. pelagic larval duration, larval behaviour) and differences in the evolutionary history of each species (e.g. distinct times and routes of colonisation, possible secondary contact events, etc.) are likely to be responsible for such patterns. Additionally, the particular environmental characteristics associated to each species preferred habitat (i.e. *P. aspera* is mainly a subtidal species and *P. candei* is mostly distributed at mid-shore levels) may have also been accountable in determining the within-species differentiation observed; it is possible that *P. aspera* has found more underwater habitat (i.e. shallow seamounts) during its evolution so as to allow a more efficient stepping stone between Madeira and Canaries, which could explain the apparent lack of genetic structure between the two archipelagos. Moreover, in contrast to the relatively stable conditions that *P. aspera* individuals experience underwater, *P. candei* habitats are more diverse, complex and heterogeneous, exposed to a large range of climatic conditions; to cope with such conditions, adaptive processes associated with niche differentiation are more likely to have happen in *P. candei* than in *P. aspera*. Indeed, the existence of two distinct habitat-related morphs of *P. candei* (referred for the Azores: the 'fly limpet' and the 'smooth limpet' lend support to this argument. Indeed, adaptive differentiation among marine invertebrates is not uncommon (Hauser and Carvalho 2008), with populations often evolving varying tolerances over a broad range of spatial scales to alongshore gradients of environmental factors that include salinity and pollution (e.g. Sokolova and Boulding 2004; Untersee and Pechenik 2007), vertical stresses associated to the intertidal zone (e.g. Janson 1982; Schmidt *et al.* 2000; Pardo and Johnson 2005), and wave exposure gradients (e.g. Struhsaker 1968; Kirby *et al.* 1994).

Although geographical distance *per se* can have possibly influenced differentiation of both *P. aspera* and *P. candei* throughout the Macaronesia, the influence of other processes such as the historical shifting of ocean circulation (Haug and Tiedemann 1998), the current oceanographic complexity (Johnson and Stevens 2000) as well as mesoscale variability across the region (Barton 2001) cannot be discarded and may have played an important role in limiting gene exchange among archipelagos, both at historical and contemporary scales. Other factors affecting larval development and dispersion may have also acted to produce the observed differentiation among archipelagos. For instance, at certain temperatures the final stage of larval development before metamorphosis in *P. candei* can be achieved in just 3 days upon fertilization (see Chapter 7) suggesting that the putative pelagic larval duration (PLD) may not be fully realized, and that larvae may be unable to reach far distant-populations (i.e. among archipelagos); larval longevity is still undetermined but may be shorter than expected considering other patellid limpets (i.e. studies in other patellids indicate a PLD up to 27 days; see Ribeiro 2008). Moreover, a considerable number of larvae may be retained near spawning sites due to behavioural preferences, with larval dispersion only occurring during weather extremes acting at a regional scale (i.e. within archipelago). Significant barriers to gene flow, either biological or physical-induced, are likely to have prompted isolation among archipelagos allowing evolution to take its own course, free of influence from other areas, resulting in the allopatric divergence of populations (Losos and Ricklefs 2009). The *P. candei* complex seems to constitute a good example of such common form of speciation (Faria *et al.* 2017a).

Although the number of studies addressing marine population connectivity has grown significantly in the past decades, there are still many uncertainties about the extent to which larvae are able to disperse among populations. This is mostly a consequence of the difficulty in tracking larvae in such an open system that is vast and complex. Although tags can be applied to some species to provide a means to directly measure larval movement (e.g. Jones *et al.* 1999; Almany *et al.* 2007), there is still much to be done and understood about processes and factors affecting dispersal, population structuring and connectivity. Understanding how population genetic structure is formed and evolves in space and time is therefore a challenging task and often restricted by our ability to fully address the complexity and interactive nature of all processes involved. The integration of genetic data with biological, ecological and environmental information, as provided by this thesis, offers better insights into the evolutionary mechanisms that have led species to their current distributions, pinpointing aspects about their dynamics, perseverance and survival in time.

Recruitment and the influence of environmental variables

There is no doubt that the rate of input of new individuals into population is a key aspect of population dynamics, especially when considering marine exploited species, where population persistency follows a thin balance between individuals that are removed by harvesting and individuals that are added to the population via recruitment. Clearly not all individuals arriving at a recruitment habitat will have equal chances of surviving. Instead individual recruit performance will depend on dispersal histories and carry-over effects leading to intra-specifically variable phenotypes that may or may not reach reproductive stages (for overview see Caley *et al.* 1996).

The monitoring programme, spatially and temporally replicated, performed in Chapter 6, allowed to assess the levels of recruitment of the exploited limpet *P. candei* in Azores. Recruitment was recorded throughout the year but its intensity varied in space and time. In general, a marked peak in recruitment occurred during winter/spring months, when sea surfaces temperatures were lower and wave turbulence higher. In fact, there was a significant correlation between limpet recruitment and sea surface temperature, significant wave height, wave period and wave direction. While each of these environmental variables may have influenced recruitment on its own, significant wave height was probably the most important factor triggering the recruitment of *P. candei*, since it was the only variable that accounted for inter-annual variation in recruitment levels. Additional sampling over longer time frames will likely support these findings and may help identify long-term patterns of recruitment variation and how they can be influenced by future and predicted environmental changes associated to global change. Moreover, in this study patterns of recruitment were restricted to four locations in one island; if possible, future research should include the monitoring of recruits and reproductive traits at several locations across the region (including other islands) to detect synchronised and/or asynchronous events. This would highlight aspects related to metapopulation processes and source-sink dynamics among islands and/or subpopulations within each archipelago. Given that the viability of a population may depend on surrounding populations, this information would greatly benefit the delineation of conservation strategies and reserve spatial planning (Akçakaya *et al.* 2007).

Temperature influence in limpet larval development

Not surprisingly, temperature was found to influence the development in *P. candei* larvae (see Chapter 7) and therefore changes in ocean temperature will likely have an impact on larval dispersal and population connectivity. Populations will become more dependent on local recruitment for replenishment, and thus more vulnerable to severe population fluctuations and extinction (Eckert 2003); first, rising sea temperature will promote the shortening of pelagic larval duration, which in turn may reduce the time larvae spend on the water column and hence reduce the degree of larval exchange among populations; secondly, further rises in sea temperature may cause massive larval mortality and, eventually, prevent replenishment of the populations. Moreover, the 'preference' for lower temperatures is in agreement with the fact that *P. candei* in Azores is a winter-breeder recruiting mostly during the colder period of the year (see Chapters 6 and 7).

From an evolutionary perspective, and given the above mentioned influence of temperature in larval development, global warming since the last glacial period may have led to shorter larval development times and thus greater separation of limpet stocks in the Macaronesia among the various archipelagos, effectively promoting the isolation of archipelagos. Except for populations of *P. aspera* in Madeira and Canaries, this hypothesis would be in agreement with the genetic structure detected across Macaronesia.

Future research should be conducted not only on *P. candei* but also on *P. aspera* and test the response of each species' larval development under isolated and combined effects of multiple abiotic stressors. This would provide valuable knowledge, which could be used to refine current and future transport models for limpets in Macaronesia under distinct environmental conditions. Moreover, an adequate optimization of rearing conditions is needed to fully quantify the PLD in both *P. candei* and *P. aspera*.

Implications for conservation and management

This PhD thesis highlights the complex nature of oceanic island connectivity and historical and contemporary factors associated with it, while attempting to delineate adequate management units for the conservation of highly exploited coastal invertebrates, such as *P. candei* and *P. aspera* in Macaronesia. It is known that conservation planning and species management can operate at many levels, from whole ecosystems and communities down to individual organisms. At any one of these levels, genetic approaches have proven to be powerful tools for revealing ecologically relevant insights into marine population dynamics and informing conservation approaches and strategies. In fact, over the last decades, the use of genetic data in conservation has become an important and expanding discipline with a strong theoretical framework. Yet, combining genetic data with information about the ecology, life-history traits and behavioural patterns of a species can provide a better and holistic understanding of population processes and how they influence each other (see Lowe and Allendorf

2010). This can greatly assist the development of management and conservation strategies to safeguard current stocks.

So, are limpets resilient enough to overcome fishing pressure, given their oceanic isolation status? Unlikely! Especially for *P. aspera*, which is highly harvested. It is also a protandrous hermaphrodite (Martins *et al.* 1987) and collection is largely biased towards the larger (hence mostly females) individuals; this size-selective harvesting can affect many different aspects of the life history and ecology of the species (e.g. Fenberg and Roy 2012) and has the potential to drastically skew sex ratios (e.g. McGovern *et al.* 1998), sizes at sex change (e.g. Hawkins and Roberts 2004; Rivera-Ingraham *et al.* 2011), population size structure (e.g. Branch 1975; Lasiak 1993; Branch and Moreno 1994; Sagarin *et al.* 2007) and affect *P. aspera* reproductive output and maintenance of populations (e.g. Harding *et al.* 2007). Given its isolated status, if new individuals, or gene material for that matter, are not provided from elsewhere, local extinction is definitely likely. Harvesting-induced changes to the genetic pool of the species (e.g. reduction in genetic diversity) are likely to become a major disadvantage in the face of any environmental fluctuations. Moreover, recent demographic data suggests that populations in many of the islands are already at very low sizes and if protective measures are not put in place, stocks will soon run out. It is likely that healthy and small pockets of individuals at sites inaccessible by humans may exist, but the genetic and ecological consequences of the intensive size-selective harvesting that is taking place may lead to local extinctions and ultimately affect the survival of the species (Fenberg and Roy 2008). Also, space availability is a major concern in *P. aspera*, which is mostly distributed at very low levels in the shore down to 20 m depths; here, competition for space, especially due to greater proliferation of macroalgae, limits the capacity of *P. aspera* populations to settle and recruit (Boaventura *et al.* 2002, Martins *et al.* 2008). This effect is further intensified if limpets and other herbivores (e.g. sea urchins) are removed or absent (see Coleman *et al.* 2006). Glints of hope for *P. aspera* populations do exist. For instance, a recent hypothesis suggests that males are compensating the removal of larger females by undergoing sex change earlier and presumably at smaller sizes (Martins *et al.* 2017); although this adaptive and resilient response can balance the sex-ratio of the species, the amount of gametes released into the water is drastically reduced with obvious negative consequences for the reproductive output of *P. aspera* (see Faria *et al.* 2017b).

As for *P. candei*, although the isolated status of populations from each archipelago may naturally threaten their survival (Frankham 1998), current information regarding its life-history traits and field observational data suggests that *P. candei* is likely to withstand most environmental and anthropogenically-induced threats. First, *P. candei* is not as intensively harvested as *P. aspera*; secondly, *P. candei* is a gonochoric species and individuals are known to reproduce at smaller sizes than *P. aspera*, and thus avoid any size-selective harvesting consequences; finally, recruitment is mostly occurring on the upper eulittoral where space pre-emption is less likely compared to lower shore levels. Overall, the opportunistic behaviour of *P. candei* is likely to provide the species the necessary resilience to face harvesting pressures and environmental changes.

So, what recommendations can be made to promote better sustainable practices in limpet harvesting? At the very least, conservation actions should follow the stock delimitation that is suggested by the genetic data from this thesis, with two independent stocks of *P. aspera* (Azores and Madeira plus Canaries), and three stocks for *P. candei*, one per archipelago. Nevertheless, given that genetic methods alone provide little information on demographic connectivity (Lowe and Allendorf 2010; Hawkins *et al.* 2016), and that limpet populations in most islands seem rather depleted, immediate actions should take a more conservative approach and consider each island particular population dynamics together with regional stock delimitations. No-take marine reserves should be put in place across islands and given that surveillance cannot be everywhere at any time, at least one of such areas per island should be constantly surveyed and monitored. For instance, the marine protected area (MPA) that includes the inner Caldeira of Monte da Guia at Faial island, Azores, which is a no-take and no-entry area and highly surveyed by local authorities, constitutes a tantalizing indication of what rocky shores would be if limpets were not heavily exploited (Vale 2016).

Indeed, these no-take marine reserves are increasingly regarded as effective tools for the management and conservation of marine ecosystems. Evidence of the positive effects of MPAs on the structure of exploited (although not exclusively so) populations is accumulating in the scientific literature (Halpern 2003; Sciberas *et al.* 2015). At population levels, these include increases in abundance, size and reproductive output as well as the restoration of population structure and sex-ratios of targeted species. A successful MPA, however, is one that not only protects the species within, but also functions as source/provider of larvae and adults (spill-over effect) to adjacent areas thus contributing to population maintenance and genetic diversity at broader spatial scales.

Additionally, current legislation about seasonal fishing closures, minimum legal catch sizes and limpet protection zones should be re-evaluated (for more specific details see Faria *et al.* 2017a, 2017b). For instance, in the Azores, the most recent legislation regulating the amount of limpets collected by qualified catcher is of 80 kg per day of fresh-living weight (Portaria nº 73/2015). This seems an excessive amount; for instance, let us consider the number of licensed catchers in S. Miguel island in 2014 (16 catchers), the duration of the harvesting season (153 days), the average mass of individuals with at least the minimum size allowed for harvesting (~ 15 g); and the total area where limpets may virtually occur throughout the island (9.43×10^5 m^2; excluding protected areas and considering that *P. aspera* is mostly distributed from the very low shore to 5 m depth); if licensed individuals do max-out their catching limits, then each m^2 should have at least a total of 14 limpets with the minimum size for harvesting (45 mm). Although this is a very rough estimate, as there are no scientific and spatially-explicit data regarding the abundance of *P. aspera* below low tide levels, observational evidence suggest that this threshold is well beyond current abundances on most rocky shores of São Miguel island. Overall, the (arbitrary?) limit set by the current legislation seems counter-productive and does not support the sustainable use of such ecological and economical important resource in the region.

Moreover, stock integrity becomes further at risk due to illegal poaching and by recreational harvesters that are allowed to collect 1.5 kg of limpets in the intertidal zone during weekends and holidays. Updated and adequate population census and studies concerning the life-history, size structure and

growth patterns of limpets in Macaronesia are therefore urgently needed. For instance, a well-performed study of *P. aspera* stock integrity in Madeira by Sousa *et al.* (2017) suggests that the species is currently under-exploited in the island. In comparison to Azores, harvesting regulations seem more restrictive; the number of licenses and daily capture quantities are more limiting than those applied in Azores. Although in the short-term, these regulations may be preventing *P. aspera* stock in Madeira from over-exploitation, the high demand for such resource in Azores (i.e. limpets caught in Madeira are often sold in the Azores), the low frequency of older individuals in the natural populations in Madeira (Sousa *et al.* 2017), and the lack of knowledge on connectivity processes among nearby islands and archipelagos, may negatively affect the susceptibility of the stock to fishing pressure.

Overall, improving the scientific basis of stock identification and assessment requires the integration of a broad spectrum of complementary techniques e.g. genetic methods, morphometrics, catch data, life-history characteristics, recruitment and population census, etc. (see Begg and Waldman 1999). These should allow a more accurate determination of stock structure and populations dynamics of exploited species, a vital aspect for designing appropriate management and conservation regulations in fisheries.

Limitations and Future Perspectives

This study would greatly benefit from a more extensive sampling approach, particularly with the inclusion of individuals/ populations from all islands across the Macaronesia where *P. candei* and *P. aspera* occur. This would be especially important in the case of Selvagens, which are located between Madeira and Canaries archipelagos and though to hold some of the older/ancestral forms of *Patella* in the Macaronesia region. Not only this would strengthen our understanding of genetic population structure and connectivity processes, but would also provide a better insight into the evolutionary history of both *P. candei* and *P. aspera* across the region. Unfortunately, due to logistics constraints no such sampling could be performed for this thesis.

To increase our understanding about population dynamics, stock integrity and the identification of historical, environmental and biological factors influencing metapopulation connectivity, future research should follow a multidisciplinary approach by pairing genetic tools with more detailed information on larval and recruitment sampling, behavioural studies, natural tags and oceanographic simulation. For each species and considering the genetic relationship among archipelagos detected in this thesis, future work should be aimed at:

- Increasing the spatial scale detection of recruitment levels across the region;
- Testing larval development under distinct and combined environmental factors;
- Combining modules of biological and physical nature to simulate and model larval transport in its natural environment, following its life-history traits, assessing at the same time the role of transport in regulating population connectivity, and the role of different biological and physical factors on limpet dispersal;

- Establishing yearly census programs to access the structure and demography of populations;
- Investigating the role of limpet gene expression and transcriptome responses in adaptation to the environmental particularities of each archipelago (i.e. thermal tolerance).

Above all, and given the endemic nature of *P. candei* and *P. aspera* and their isolation status within the NE Atlantic archipelagos, stock conservation should follow an integrated management approach, provided that stock management authorities and scientific community work together to find the best way to protect and guarantee the sustainable use of natural resources (i.e. limpets) across archipelagos. More generally, mitigating the effects of harvesting would require us to shift from management strategies that are designed to maximize yield (Longhurst 2006) to those that can preserve the natural variations that characterize species and ecosystems.

References

Akçakaya HR, Mills G, Doncaster CP (2007) The role of metapopulations in conservation. In *Key Topics in Conservation Biology*. Macdonald DW, Service K (Eds). Blackwell Publishing, Oxford, UK. pp. 64-84.

Allendorf FW, Hard JJ (2009) Human-induced evolution caused by unnatural selection through harvest of wild animals. *Proceedings of the National Academy of Sciences of the USA* 106: 9987-9994.

Almany GR, Berumen ML, Thorrold SR, Planes S, Jones GP (2007) Local replenishment of coral reef fish populations in a marine reserve. *Science* 316: 742-744.

Barton ED (2001) Ocean currents: Atlantic Eastern Boundary - Canary Current/Portugal Current. In *Encyclopaedia of Ocean Sciences*. Volume 1. Steele J, Thorpe S, Turekian K (Eds). Academic Press, London. pp. 380-389.

Begg GA, Waldman JR (1999) An holistic approach to fish stock identification. *Fisheries Research* 43: 35-44.

Boaventura D, Alexander M, Santina PD, Smith ND, Ré P, Fonseca LC, Hawkins SJ (2002) The effects of grazing on the distribution and composition of low-shore algal communities on the central coast of Portugal and on the southern coast of Britain. *Journal of Experimental Marine Ecology and Biology 2*67: 185-206.

Branch G, Moreno C (1994) Intertidal and subtidal grazers. In *Rocky Shores: Exploitation in Chile and South Africa*. Siegfried R (Ed). Springer-Verlag, Berlin. pp. 75-100.

Branch GM (1975) Mechanisms reducing intraspecific competition in *Patella* spp.: migration, differentiation and territorial behaviour. *Journal of Animal Ecology* 44: 575-600.

Caley MJ, Carr MH, Hixon MA, Hughes TP, Jones GP, Menge BA (1996) Recruitment and the local dynamics of open marine populations. *Annual Review of Ecology and Systematics* 27: 477-500.

Coleman RA, Underwood AJ, Benedetti-Cecchi, Aberg P, Arenas F, Arrontes J, Castro J, Hartnoll RG, Jenkins SR, Paula J, Santina PD, Hawkins SJ (2006) A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia* 147: 556-564.

Conover DO (1998) Local adaptation in marine fishes: evidence and implications for stock enhancement. *Bulletin of Marine Science* 62: 477-493.

Díaz-Viloria N, Cruz P, Próo SAG, Perez-Enriquez R (2009) Genetic connectivity among pink abalone *Haliotis corrugata* populations. *Journal of Shellfish Research* 28: 599-608.

Eckert GL (2003) Effects of the planktonic period on marine population fluctuations. *Ecology* 84: 372-383.

Eldridge MDB, King JM, Loupis AK, Spencer PBS, Taylor AC, Pope LC, Hall GP (1999) Unprecedented low levels of genetic variation and inbreeding depression in an island population of the black-footed rock-wallaby. *Conservation Biology* 13: 531-541.

Faria J, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Pita A, Neto AI, Presa P (2015) A new multiplexed microsatellite tool for metapopulation studies in the overexploited endemic limpet *Patella aspera* (Röding, 1798). *Animal Genetics* 46(1): 96-97.

Faria J, Pita A, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Neto AI, Presa P (2016) A multiplex microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian archipelagos. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 775-781.

Faria J, Martins GM, Pita A, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017a) Disentangling the genetic and morphological structure of *Patella candei* complex in Macaronesia (NE Atlantic). *Ecology and Evolution* 7(16): 6125-6140.

Faria J, Pita A, Martins GM, Ribeiro P, Hawkins SJ, Presa P, Neto AI (2017b) Inbreeding in the exploited limpet *Patella aspera* across the Macaronesia archipelagos (NE Atlantic): implications for conservation. *Fisheries Research* 198: 180-188.

Fenberg PB, Roy K (2008) Ecological and evolutionary consequences of size-selective harvesting: how much do we know?. *Molecular Ecology* 17: 209-220.

Fenberg PB, Roy K (2012) Anthropogenic harvesting pressure and changes in life history: insights from a rocky intertidal limpet. *American Naturalist* 180(2): 200-210.

Frankham R (1996) Relationship of genetic variation to population size in wildlife. *Conservation Biology* 10: 1500-1508.

Frankham R (1997) Do island populations have less genetic variation than mainland populations?. *Heredity* 78: 311-327.

Frankham R (1998) Inbreeding and extinction: Island populations. *Conservation Biology* 12: 665-675.

Goldstien SJ, Gemmell NJ, Schiel DR (2009) Colonization and connectivity by intertidal limpets among New Zealand, Chatham and Sub-Antarctic Islands. I. Genetic connections. *Marine Ecology Progress Series* 388: 111-119.

Halpern BS (2003) The impact of marine reserves: do reserves work and does reserve size matter?. *Ecological Applications* 13: S117-S137.

Harding JM, Mann R, Kilduff C (2007) The effects of size on fecundity in a large marine gastropod *Rapana venosa*. *Journal of Shellfish Research* 26: 33-42.

Haug GH, Tiedemann R (1998) Effect of the formation of the Isthmus of Panama on Atlantic Ocean thermohaline circulation. *Nature* 393: 673-678.

Hauser L, Adcock GJ, Smith PJ, Ramírez JHB, Carvalho GR (2002) Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *Proceedings of the National Academy of Sciences of the USA* 99(18): 11742-11747.

Hauser L, Carvalho G (2008) Paradigm shifts in marine fisheries genetics: ugly hypotheses slain by beautiful facts. *Fish and Fisheries* 9: 333-362.

Hawkins JP, Roberts CM (2004) Effects of fishing on sex-changing Caribbean parrotfishes. *Biological Conservation* 115: 213-226.

Hawkins SJ, Bohn K, Sima DW, Ribeiro P, Faria J, Presa P, Pita A, Martins GM, Neto AI, Burrows MT, Genner MJ (2016) Fisheries stocks from an ecological perspective: Disentangling ecological connectivity from genetic interchange. *Fisheries Research* 179: 333-341.

Hellberg ME (2009) Gene flow and isolation among populations of marine animals. *Annual Review of Ecology, Evolution, and Systematics* 40: 291-310.

Janson K (1982) Genetic and environmental effects on the growth rate of *Littorina saxatilis*. *Marine Biology* 69: 73-78.

Johnson J, Stevens I (2000) A fine resolution model of the eastern North Atlantic between the Azores, the Canary Islands and the Gibraltar Strait. *Deep-Sea Research I* 47: 875-899.

Jones GP, Millicich MJ, Emslie MJ, Lunow C (1999) Self-recruitment in a coral reef fish population. *Nature* 402: 802-804.

Kirby RR, Bayne BL, Berry RJ (1994) Phenotypic variation along a cline in allozyme and karyotype frequencies, and its relationship with habitat, in the dog-whelk *Nucella lapillus* (L.). *Biological Journal of the Linnean Society* 53: 255-275.

Lamy T, Pointier JP, Jarne P, David P (2012) Testing metapopulation dynamics using genetic, demographic and ecological data. *Molecular Ecology* 21: 1394-1410.

Lasiak T (1993) Temporal and spatial variations in exploited and non-exploited populations of the intertidal limpet *Cellana capensis*. *Journal of Molluscan Studies* 59: 295-307.

Longhurst A (2006) The sustainability myth. *Fisheries Research* 81: 107-112.

Losos JB, Ricklefs RE (2009) Adaptation and diversification on islands. *Nature* 457: 830-836.

Lowe WH, Allendorf FW (2010) What can genetics tell us about population connectivity?. *Molecular Ecology* 19: 3038-3051.

Martins HR, Santos RS, Hawkins SJ (1987) Exploitation of limpets (*Patella* spp.) in the Azores with a preliminary analysis of the stocks. *ICES Report*, 1987/K 53: 1-17.

Martins GM, Jenkins SR, Hawkins SJ, Neto AI, Thompson RC (2008) Exploitation of rocky intertidal grazers: population status and potential impacts on community structure and functioning. *Aquatic Biology* 3: 1-10.

Martins GM, Borges CDG, Vale M, Ferraz RR, Martins HR, Santos RS, Hawkins SJ (2017) Exploitation promotes earlier sex change in a protandrous patellid limpet, *Patella aspera* Röding, 1798. *Ecology and Evolution* 7(10): 3616-3622.

McGovern JC, Wyanski DM, Pashuk O, Manooch CS, Sedberry GR (1998) Changes in the sex ratio and size at maturity of gag, *Mycteroperca microlepis*, from the Atlantic coast of the southeastern United States during 1976-1995. *Fishery Bulletin* 96: 797-807.

Pardo LM, Johnson LE (2005) Explaining variation in life-history traits: growth rate, size, and fecundity in a marine snail across an environmental gradient lacking predators. *Marine Ecology Progress Series* 296: 229-239.

Perez M, Branco M, Llavona A, Ribeiro PA, Santos AM, Hawkins SJ, Dávila JA, Presa P, Alexandrino P (2007) Development of microsatellite loci for the black-footed limpet, *Patella depressa*, and cross-amplification in two other *Patella* species. *Conservation Genetics* 8: 739-742.

Pinsky ML, Palumbi SR (2014) Meta-analysis reveals lower genetic diversity in overfished populations. *Molecular Ecology* 23: 29-39.

Portaria nº 73/2015. S.R. do Mar, Ciência e Tecnologia. Região Autónoma dos Açores. Portugal.

Ribeiro PA, Branco M, Hawkins SJ, Santos AM (2010) Recent changes in the distribution of a marine gastropod, *Patella rustica*, across the Iberian Atlantic coast did not result in diminished genetic diversity or increased connectivity. *Journal of Biogeography* 37(9): 1782-1796.

Ribeiro PA (2008) *Dispersal and connectivity of northeastern Atlantic patellid limpets: a multidisciplinary approach*. PhD thesis, University of Southampton.

Rivera-Ingraham GA, Espinosa F, García-Gómez C (2011) Environmentally mediated sex change in the endangered limpet *Patella ferruginea* (Gastropoda: Patellidae). *Journal of Molluscan Studies* 77: 226-231.

Sagarin RD, Ambrose RF, Becker BF, Engle JM, Kido J, Lee SF, Miner CM, Murray SN, Raimondi PT, Richards D, Roe C (2007) Ecological impacts on the limpet *Lottia gigantea* populations: human

pressure over a broad scale on island and mainland intertidal zones. *Marine Biology* 150: 399-413.

Sanford E, Kelly MW (2011) Local adaptation in marine invertebrates. *Annual Review of Marine Science* 3: 509-535.

Schmidt PS, Bertness MD, Rand DM (2000) Environmental heterogeneity and balancing selection in the acorn barnacle *Semibalanus balanoides*. *Proceedings of the Royal Society of London B* 267: 379-384.

Sciberras M, Jenkins SR, Mant E, Kaiser MJ, Hawkins SJ, Pullin AS (2015) Evaluating the relative conservation value of fully and partially protected marine areas. *Fish and Fisheries* 16: 58-77.

Smith PJ, Francis RICC, McVeagh M (1991) Loss of genetic diversity due to fishing pressure. *Fisheries Research* 10: 309-316.

Sokolova IM, Boulding EG (2004) A neutral DNA marker suggests that parallel physiological adaptations to open shore and salt marsh habitats have evolved more than once within two different species of gastropods. *Marine Biology* 145: 133-147.

Sousa R, Delgado J, Pinto AR, Henriques P (2017) Growth and reproduction of the north-eastern Atlantic keystone species *Patella aspera* (Mollusca: Patellogastropoda). *Helgoland Marine Research* 71:8. https://doi.org/10.1186/s10152-017-0488-9.

Struhsaker JW (1968) Selection mechanisms associated with intraspecific shell variation in *Littorina picta* (Prosobranchia: Mesogastropoda). *Evolution* 22: 459-480.

Untersee S, Pechenik JA (2007) Local adaptation and maternal effects in two species of marine gastropod (genus *Crepidula*) that differ in dispersal potential. *Marine Ecology Progress Series* 347: 79-85.

Vale M (2016) *Influence of climate change and other impacts on rocky intertidal communities of the Azores.* PhD thesis, University of Southampton.

Walsh MR, Munch SB, Chiba S, Conover DO (2006) Maladaptive changes in multiple traits caused by fishing: impediments to population recovery. *Ecology Letters* 9: 142-148.